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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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THE RELATIONSHIP BETWEEN THE GROWTH CAPACITY OF PRIMARY TUMORS AND THE DISTRIBUTION OF METASTASES OF A TRANSPLANTED TUMOR OF THE RABBIT.

By CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, March 2, 1925.)

In connection with observations in this laboratory upon rabbits with an experimentally transmitted malignant disease, and as an aid to statistical study, an attempt has been made to develop an index of malignancy whereby a numerical estimate may be placed upon the severity of the disease. It was found necessary to employ a double index consisting of two figures, one referring to the primary tumor and the other to metastases. The primary tumor was evaluated on a volume basis and the metastases according to distribution. It was felt that as regards the primary tumor, the measurement of volume can be expected to indicate quite precisely its capacity for local development, and the number of metastases would signify the capacity of the tumor cells to gain access to and survive in distant organs. These capabilities are fundamental qualities denoting malignancy.

A very considerable number of tumor-bearing rabbits has now been dealt with by means of the index, and it has been interesting to note the close agreement between the evaluation of malignancy as rendered by the two figures of the index taken together and the impression derived from the frequent clinical observations and the autopsy records. The data obtained have proved of assistance in tracing variations that occur in disease severity among individuals and groups.

Not only do the indices lend themselves well to the study of the variations in the severity of the disease as a whole, but, taken separately, they furnish a quantitative expression of the relationship that exists between the two components of the malignant process, the pri-

mary tumor and the metastases. It is the purpose of the present paper to approach the subject from the latter standpoint. The accumulated material will be examined with particular reference to the indices of malignancy, and an endeavor made to arrive at conclusions concerning the relationship existing between the growth capacity of the primary tumor and the distribution of metastases.

EXPERIMENTAL.

Material.—The tumor which forms the basis for this study arose spontaneously in the scrotal skin of a rabbit being used for other experimental purposes, and proved fatal to that animal. We have preserved the tumor by successive intratesticular transplantations in rabbits. The reader is referred to previous publications (1–6) for detailed descriptions of the nature of the tumor (epithelioma) and of the disease which it produces. Suffice it to say here that it is highly malignant and metastasizes widely by way of the lymphatics and blood stream. The disease is strikingly similar to the malignant cancer of man, but differs therefrom in a much more rapid course and in the frequent occurrence of spontaneous recovery.

A group of 128 rabbits was used for this study. Among these were some of all common breeds and of ages ranging from 6 months to 3 or 4 years. Caging and feeding routine was uniform throughout. Each animal was inoculated into the right testicle with 0.3 cc. of a tumor cell emulsion prepared from tissue freshly removed from stock tumor rabbits. 6 days after inoculation, a time when the cell emulsion had given rise to a palpable primary tumor in all rabbits, measurement was made of the tumor in two dimensions with a vernier calipers. From these diameters the volume was calculated approximately, expressed in c.mm. The volume of the primary tumor was obtained in this way every 3 days thereafter. Rabbits moribund with the disease were killed with ether anesthesia and autopsied; also, all rabbits surviving to 60 days were killed and autopsied. At autopsy a count was taken of the number of organs presenting gross metastases, a uniform basis of counting being used in all cases, and this figure was recorded as representing the distribution of metastases.

The group of 128 rabbits represented three entire experimental series. Series 1 consisted of 67 rabbits, inoculated Nov. 15, 1923; Series 2 consisted of 30 rabbits, inoculated Jan. 17, 1924; and Series 3, 31 rabbits, inoculated Mar. 1, 1923. Taken as a whole, Series 1 presented clinically a relatively low grade of malignancy, Series 2 a fairly high grade of malignancy, and Series 3 a malignant behavior of extreme degree. 54 of the rabbits were not interfered with in any way besides the tumor inoculation, but the remaining animals were subjected, in addition to this, to a variety of artificially imposed conditions. Thus, 27 rabbits were thyroidectomized a few days before inoculation, 12 were partially

thyroidectomized, 25 were thymectomized, and 5 were inoculated with embryonic rabbit tissues prior to inoculation with the tumor.

Method of Analysis.—Separate analysis has been made of each series of rabbits for the reason that the series were run at different times of the year, were inoculated with tumor cells from different stock sources (although of like variety), and displayed malignant behavior of quite different degrees. Each series is analyzed graphically by means of six charts. The charts for Series 1 are assembled in Text-fig. 1, those for Series 2 in Text-fig. 2, and those for Series 3 in Text-fig. 3. In each chart every rabbit of the series is represented separately by a column erected upon a base line; the position of the column on the base line is determined by the size of the primary tumor (expressed in c.mm.), and the height of the column is determined by the distribution of the metastases found at autopsy (expressed in the number of organs involved). The charts for each series differ from one another only as regards the time at which the size of the primary tumor was measured. That is, Chart 2 is based on the size attained by the tumor on the 15th day after inoculation, Chart 3 on the size at the 21st day, Chart 4 at the 33rd day, Chart 5 at the 40th day, and Chart 6 at the 60th day. Chart 1, on the other hand, is based on the maximum size reached by the primary tumor, regardless of when that size was determined. The individual column heights remain, of course, unaltered from chart to chart.

By means of this system it is possible to examine the relationship existing between the magnitude of the primary tumor at any one time and the ultimate distribution of metastases from it.

Results.

Individual Behavior.—A general survey of the charts brings forth at once two striking facts. In the first place, the severity of the neoplastic disease as a whole is extremely variable. Secondly, the relation between the primary and secondary phases of the disease, as indicated by the relation of the heights of the columns to their positions on the base line, is also extremely variable. That is, animals possessing primary tumors of equal size differ greatly in degree of involvement by metastases; and, conversely, animals with approximately equal metastatic involvement frequently display marked disparity in the size of the primary tumor. A few specific instances drawn from the charts will serve as illustrations.

In Text-fig. 1, Chart 2, Rabbit A possesses a primary tumor measuring 500 c.mm., and Rabbit B a tumor of 410 c.mm. If these two cases are followed through the charts it will be found that their primary tumors enlarge at approximately the same rate, so that at the end of the experimental period (Chart 6)

the tumor of the first measured 1,580 c.mm. and the tumor of the second 1,590 c.mm. Nevertheless, at autopsy the metastatic involvement found in Rabbit B was six times as severe as that in Rabbit A.

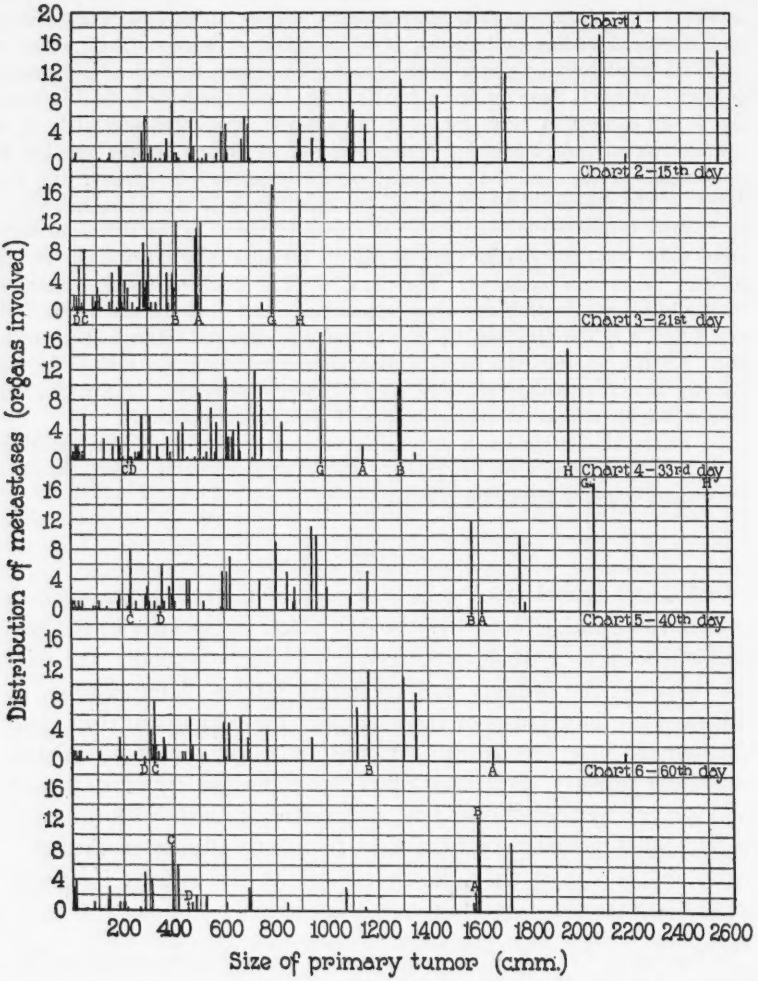
Chart 1
Chart 2–15th day
Chart 3–21st day
Chart 4–33rd day
Chart 5–40th day
Chart 6–60th day
Distribution of metastases (organs involved)
0 4 8 12 16 20
200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600
Size of primary tumor (cmm.)

TEXT-FIG. 1. Relation of size of primary tumor to distribution of metastases—Series 1.

In the same series (Text-fig. 1) are Rabbits C and D. The primary tumors of these animals developed much more slowly than those of the pair just mentioned and at the end of the experiment measured 390 and 450 c.mm. respectively.

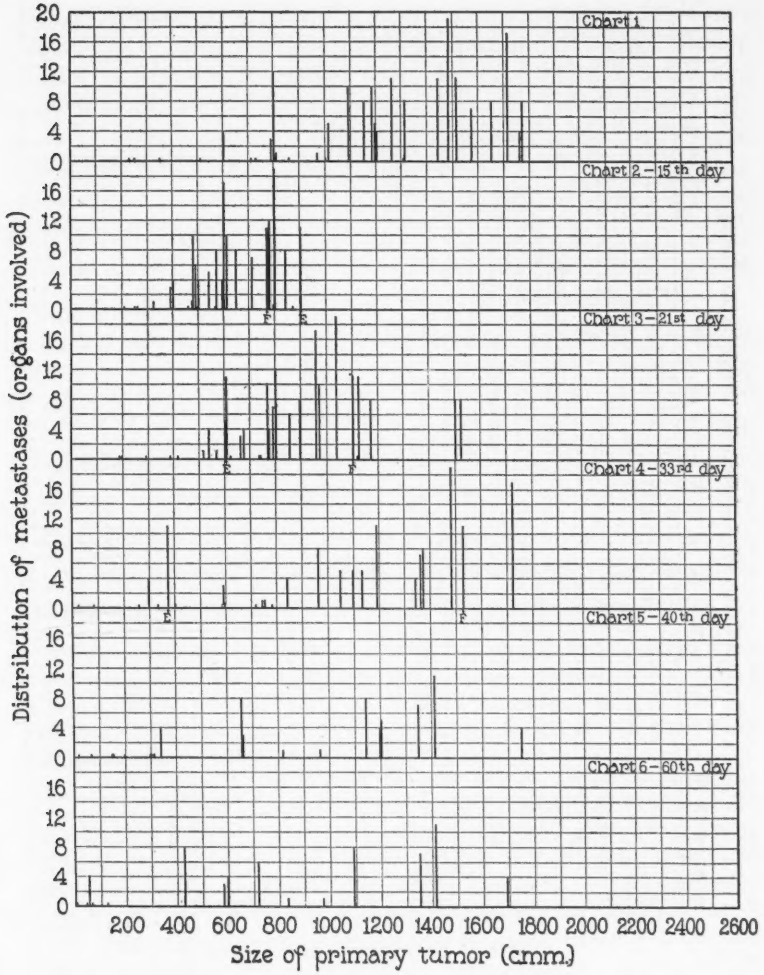
Chart 1
Chart 2 – 15th day
Chart 3 – 21st day
Chart 4 – 33rd day
Chart 5 – 40th day
Chart 6 – 60th day
Distribution of metastases (organs involved)
0 4 8 12 16
200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600
Size of primary tumor (cmm.)

TEXT-FIG. 2. Relation of size of primary tumor to distribution of metastases—Series 2.

Rabbit C presented, at post mortem, eight organs extensively invaded by metastases and Rabbit D showed no evidence of ever having had a metastasis.

In Text-fig. 2 are two rabbits, E and F, both of which presented at autopsy

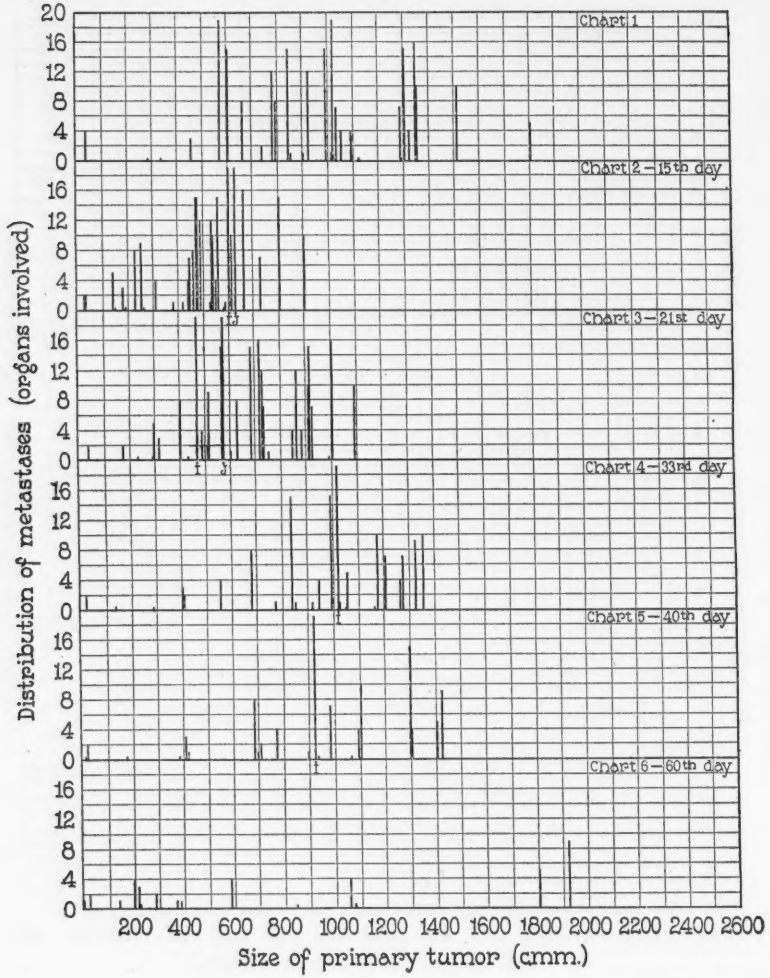
Chart 1
Chart 2—15th day
Chart 3—21st day
Chart 4—33rd day
Chart 5—40th day
Chart 6—60th day
Distribution of metastases (organs involved)
0 4 8 12 16 20
200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600
Size of primary tumor (cmm.)

TEXT-FIG. 3. Relation of size of primary tumor to distribution of metastases—Series 3.

widespread and equally extensive metastasis; yet, it will be seen from the charts that the primary tumor of the first measures only 370 c.mm. in Chart 4, while the tumor of the second in the same chart is registered at 1,530 c.mm.

This irregularity of behavior among individual animals requires that the study of group behavior be undertaken with great caution, and on this account methods have been employed for statistical analysis of the material which retain the individual elements. The use of averages with data of this sort may be very misleading. Attention will be paid in the following analysis only to certain broad tendencies that are clearly discernible in the groups of animals as they stand depicted separately upon the charts.

Group Behavior.—First, there is evidence as to the relative malignancy of the tumor in the three series of rabbits.

A rough comparison of the text-figures impresses one with the considerable differences in the severity of the metastatic involvement in the three series. Also, the proportion of animals appearing in Chart 6 of each text-figure as survivals indicates differences in mortality rate. These two manifestations support the clinical impression referred to above, that Series 1, as a whole, presented a mild form of malignancy, Series 2 a fairly high grade of malignancy, and Series 3 a malignant condition of extremely high grade.

Second, there is to be seen in the charts a tendency for the severity of metastasis to conform to certain relationships with the size of the primary tumor. These may be traced in each series as follows:

In Charts 2, 3, and 4 of Text-fig. 1 the average height of the columns is seen to increase progressively, reading from left to right. Inference: In this series of rabbits, in which the disease had a low grade malignancy, the distribution of metastases was roughly proportional throughout to the size of the primary tumor, especially in the early stages of the disease.

In Charts 2, 3, 4, and 5 of Text-fig. 2 there is likewise a steady increase in the average height of the columns, from left to right, although in Charts 2 and 3 this increase holds only up to a certain point and then a slight decrease in the heights of the columns occurs. The turning point in Chart 2 lies at about 800 c.mm., and in Chart 3 at 1,050 c.mm. Inference: In this series of rabbits, in which the disease was moderately severe, the distribution of metastases was roughly proportional to the size of the primary tumor, especially in the early stages of the disease. An exception to this rule occurred on the 15th and 21st days after inoculation, when those rabbits possessing primary tumor above certain size limits presented a tendency to an inverse relationship between the two components of the tumor process.

In Charts 2, 3, 4, and 5 of Text-fig. 3 the height of the columns increases steadily, from left to right, up to a certain point in each chart, and then decreases. The turning point in Chart 2 is at 620 c.mm., in Chart 3 at 570 c.mm., in Chart 4 at 1,020 c.mm., and in Chart 5 at 930 c.mm. Inference: In this series of rabbits, which was affected for the most part with a very severe form of the disease, the distribution of metastases was also roughly proportional to the size of the primary tumor, especially in the earlier stages of the disease. An exception to this rule occurred here, too, among those animals possessing primary tumors above certain size limits, for they presented a tendency to an inverse relationship between the two components of the disease.

From a comparison of the corresponding charts of Text-figs. 2 and 3, we may infer that the size limits at which the change in proportion took place from a direct to an inverse relationship were lower under conditions of extreme malignancy than under those of moderate malignancy.

To summarize these points, we may say that during the more active stages of the disease, the relationship between the size of the primary tumor and the distribution of metastases tends to be a direct one, unless the conditions be such that the disease is running a course of high or extreme malignancy for the series as a whole, when the direct proportion gives place to an inverse one among those rabbits with the larger primary tumors. Also, the change from the direct to the inverse relationship takes place at a level of primary tumor size that is lower the more severe the existing conditions of malignancy.

There is a third feature to be discerned in this study of mass behavior that is sufficiently well defined for mention; namely, that of differing rates of growth of the primary tumor.

In Chart 2 of each of the text-figures the upper limit of primary tumor size is the same, 900 c.mm. In Chart 3 the upper limit is quite different in the three text-figures, for in Text-fig. 1 it lies at 1,960 c.mm., in Text-fig. 2 at 1,500 c.mm., and in Text-fig. 3 at 1,090 c.mm. In Chart 4 of each series there is still greater difference in the size limits of the primary tumors; in Text-fig. 1 it is situated at 2,500 c.mm., in Text-fig. 2 at 1,700 c.mm., and in Text-fig. 3 at 1,350 c.mm. These variations in the rate of tumor growth in the three series of rabbits are brought out not only in the size limits of the primary tumors, but also in the behavior of individual primary tumors. As an example, in Text-fig. 1 the primary lesions of Rabbits G and H, the most seriously affected members of the group, showed an increase in size of 160 and 178 per cent, respectively, in the period between Charts 2 and 4; and in Text-fig. 3, of two severely diseased rabbits, the primary tumor of Rabbit I increased only 70 per cent in the same period, and that of Rabbit J actually diminished slightly in size before the death of the animal.

It is, therefore, quite strikingly demonstrated by these data, that up to the 15th day of the disease the range of size of the primary tumors in the three groups of animals was the same, and that from this point on the rate of growth of the more active tumors in each series changed from series to series inversely as did the prevailing degree of malignancy.

Little attention has been paid in this study to Charts 5 and 6 in the text-figures, and the reason for this is that at the late stages of the experiments the surviving animals are too few for the purpose of a mass analysis.

DISCUSSION AND CONCLUSIONS.

In placing a proper evaluation upon these statistical results we are assisted considerably by the uniformity of circumstances that exists as a background for the experiments.

The biological characteristics of the tumor have been carefully studied through forty generations. The initial processes of the disease are uniform in all rabbits inoculated by the routine method, to the extent that the tumor cells that are introduced begin immediately to proliferate and to invade the surrounding testicular parenchyma, including the blood and lymph vessels. As early as the 14th day of the disease microscopic examination of the peripheral tissues reveals emboli of tumor cells lying at various points in the vascular bed, *i.e.* the sinuses of the regional lymph nodes, the capillaries of the lungs, the glomerular tufts of the kidneys, etc., and there is every reason to presume that practically every rabbit harboring a primary tumor in the testicle is exposed at an early stage to an extensive intravascular dispersion of malignant cells.

The environmental conditions were the same throughout in all respects, except that the three series were run at different times.

The rabbit stock used in these experiments was selected and matched according to breed and age with great care, excepting those groups containing exclusively very old or very young rabbits. The results in these special groups, together with those which received operative interference of one sort or another before inoculation, serve to strengthen the validity of the conclusions to be drawn since they fall directly in line with the behavior of the others from the standpoint of this analysis.

Upon this background of uniformity two features in the data obtained stand out most noticeably, *i.e.* the great irregularity in the severity of the disease as a whole among individual animals, and the equally pronounced irregularity in the quantitative relationship

between the primary and secondary parts of the disease process as they manifest themselves in individuals. The first of these phenomena may be accounted for on the basis of variation in host susceptibility to the disease in its entirety. Discussion of this subject is not within the scope of the present paper. The second apparently has to do with differences in host susceptibility to the two principle divisions of the disease, for the efficiency of the mechanism of resistance against the inroads of the primary tumor evidently does not always parallel that of resistance against metastasis.

The contrast is most striking between the picture presented by one rabbit carrying an enormous tumor of the testicle and a multitude of metastases scattered through practically every organ of the body, and that of another rabbit of the same group which has a primary tumor of the same great size and yet presents not a single gross metastasis. Both of these rabbits are deficient in the ability to suppress the primary growth and both have doubtless been subjected to a widespread dissemination of tumor cell emboli. The embolic cells of the first rabbit survived and grew readily in the peripheral tissues, but the tumor emboli which were liberated into the circulation of the second rabbit have shown not the slightest signs of survival, for, not only are no peripheral tumors to be found in this animal, but minute examination of the tissues fails to discover cicatrization or cellular infiltration at points where tumor deposits are most common. One is led by these observations to the inference that the second of these rabbits, while equally as deficient as the first in the ability to check the local growth of an organized tumor, is possessed of a means of defense, not shared in equally by the other, which operates to prevent the survival of recently deposited tumor cell emboli before they have had an opportunity to invade the peripheral tissues to any degree.

If a rabbit of the type of the second animal just described be autopsied at an active stage of the disease, embolic malignant cells may be found in the peripheral capillary beds, and the great majority or all of these emboli are seen to be contained in thrombus masses. Indeed, any tumor-bearing rabbit[1] sacrificed at the 2nd or 3rd week will show microscopically a certain proportion of the recently deposited metastases still retained within the lumina of the smaller blood vessels and embedded in clot. Schmidt (7) has described a similar thrombus formation about metastatic tumor cells in human cancer. It is probable that the capsule of fibrin which immediately encloses the cells is an important factor in the front line of defense that these animals seem to possess against the survival of blood-borne tumor deposits. This thrombotic wall probably interferes more or less effectually with the implantation of the tumor cells upon the

[1] Pearce and Brown (5), page 347.

capillary endothelium, not only passively by exclusion, but also actively by strangulation of the embolus as the clot contracts in the process of coagulation. It would appear, therefore, that the normal animal organism is equipped with a non-specific means of defense against living foreign particles in the blood stream, which is in nature little more than the embrace of the embolus in the grip of a contractile capsule of fibrin. It would follow from this, that the degree of resistance of metastasis, as far as the successful implantation of tumor cells is concerned, depends in part at least upon the integrity of the mechanism of coagulation of the blood, particularly that which has to do with the firmness and contractibility of the clot, and upon the ability of this mechanism to retain its efficiency under the strain of a progressive, debilitating disease. We have found changes in the coagulability of the blood, especially as regards clot retractility, in animals displaying an actively advancing tumor process. The studies of these changes made thus far (8) have indicated their coincidence with the development of blood-borne metastases, even as early as the 2nd week, and from other standpoints, also, an intimate connection with the phenomenon of metastasis.

Whatever the agent or agents that serve to protect the host of a malignant tumor against the development of metastases, the efficiency of the protection thus afforded is demonstrated by the evidence of the present analysis to be frequently disproportionate with that of the other defense factors possessed by the body against the tumor.

From the standpoint of group behavior the first conclusion drawn from the statistics has to do with the differences in the malignancy of the tumor displayed in the three series of rabbits. The tendency for the severity of the disease, taken as a whole, to vary from time to time has been recognized from the beginning of our observations, and a coincidence has been found with alterations in meteorological conditions. The problem is being treated in detail in other publications (9–11).

As concerns the relationship in the mass between the size of the primary tumor and the distribution of metastases it appears that, for the most part, in all three series of rabbits these two divisions of the disease were proportional. However, the relationship was found to be disturbed in certain groups of animals, for in Series 2 and 3 a group of rabbits with primary tumors above certain size limits displayed a reduced distribution of metastases. In addition, in these same groups of animals there was a reduction in the rate of growth of the primary tumor. Both manifestations were more pronounced in Series 3 than in Series 2 and disappeared altogether in Series 1.

It is not surprising to find that a direct relationship predominates between the extent of the primary and secondary lesions, for this corresponds to the rule of cause and effect. The more active the growth of the primary tumor the more abundant the dissemination of emboli and the more trying the tax upon all powers of resistance of the animal. On the other hand, the occurrence in certain groups of animals of a tendency to the reversal of this relationship and, at the same time, to reduction in the rate of growth of the primary tumor is not so easily explained. And when it is also appreciated that the reduction in the distribution of metastases and in the rate of tumor growth becomes more pronounced from series to series as the general condition of malignancy of the series increases, we appear to be confronted by a paradoxical situation, in which there occurs an inverse relationship between the extent of the lesions produced by the tumor and the degree of malignancy prevailing for the series as estimated on a clinical basis.

This paradoxical circumstance appearing in certain groups of rabbits might be explained on a basis of malnutrition, *i.e.* that accompanying these highly aggressive disease processes there is an exhaustion of available nutriment, and that consequently lesions of larger proportions are not able to develop. There may be, indeed, an element of this nature in the production of the phenomenon, but its influence is probably subsidiary, because the manifestations under consideration set in at a stage when the animals were still fairly well nourished. The manifestations were well defined and abrupt. The boundary line between this group of animals and the rest of the series was distinct; the course of the disease progressed according to expectancy up to the end of the 2nd week when the rate of the primary tumor was suddenly reduced. It has been observed, furthermore, that special symptoms and signs are likely to make their appearance in these animals; at about the 2nd or 3rd week after inoculation rabbits with rapidly growing tumors may present slight lethargy and develop a marked edematous swelling of the scrotum. The edema increases to a maximum in about 24 hours, persists for a few days, gradually subsides, and shortly thereafter the disease gives definite evidence of following one of two divergent types of behavior, either rapid recovery with necrosis and absorption of the tumor, or, after a period of retardation, quite rapid acceleration of the process to a lethal outcome. In either event the amount of tumor to be found at autopsy frequently falls short of indicating the severity of the disease Coincident with the edema there have always taken place, as far as this point has been determined, marked alterations in the coagulability of the blood, and it would appear from the concurrence of these two manifestations of physical disturbance, *i.e.* scrotal edema

and reduced blood coagulability, with the onset of the changes in the rate of growth of the primary and secondary divisions of the disease as shown in the charts, that all of these phenomena are related in some way. It would appear, also, that this group of manifestations has to do with alterations in the virulence of the disease, referable to the tumor or to the host or to both. That the host is the factor chiefly concerned in initiating these alterations of disease severity is suggested by the great variability of host resistance in this instance as compared with the uniformity of the tumor inoculum. But to explain the nature of the changes in host resistance responsible for these abrupt alterations in the disease and the symptom complex that accompanies them a great deal will have to be understood regarding the manner of control and regulation of the various agents of defense in the animal body. Nevertheless, some assistance is rendered by the data at hand. The group of manifestations in question takes place to a recognizable degree only in animals of low resistance, but not in all such animals; it takes place during the period of early rapid growth and invasion by the primary tumor, and at a time when the host is still in good physical condition, and it leads to a retardation, either temporary or permanent, of the progress of the disease. From these circumstances one would infer that the physical manifestations are of the nature of a reaction to invasion, unusual in type, and characterized by a sudden increase in host resistance. Apparently, too, the causative factor in the animal responsible for the initiation of this reaction is dependent upon some other than low resistance *per se*. Perhaps these rabbits, besides being weak in resistance to the tumor, are more sensitive to its inroads than are others of the same series, and when the stimulus of invasion has mounted to a certain critical degree of intensity, the mechanism which, it is reasonable to assume, the animal organism possesses to coordinate defense responses to the requirements of an invasion, becomes unbalanced and responds with a disproportioned, frequently maximum, defense mobilization. The immediate result of this is a reduction in tumor growth, which is temporary or permanent depending upon the efficiency of the reaction that has taken place.

This explanation of the seemingly paradoxical state of affairs that arises in certain tumor-bearing rabbits is based largely on the results of our investigations on the response to malignant disease of certain delicately balanced phenomena of blood coagulation. Report of this work will be made in the near future.

SUMMARY.

As an adjunct to the study of a transplantable malignant tumor of the rabbit an index of malignancy has been developed which has proven useful in evaluating the severity of the disease for the purpose of statistical investigations.

The index consists of two figures, one of which expresses the growth capacity of the primary lesion on a volume basis, and the other the extent of the metastatic lesions according to the distribution of peripheral tumors. In the present communication these two values for each animal are utilized for the study of the relationship existing between the size of the primary tumor and the distribution of metastases in this experimental tumor. The data from a group of 128 rabbits form the basis of the analysis. These animals represent three entire series that had been used for the purposes of other experiments at various times, all, however, conducted under carefully controlled uniform conditions. The indices of malignancy of these animals were analyzed by means of graphs, in which individual values are preserved while at the same time investigation of mass behavior is permitted.

From this analysis it is concluded (1) that the severity of the disease as a whole is very irregular among individuals of a series and from series to series, and (2) that the relationship between the extent of the primary lesions and that of the secondary lesions is inconstant among individuals but assumes definite proportions when the data are viewed in the mass. For the mass proportion between the two phases of the disease is predominatingly a direct one, but in certain small groups of seriously affected rabbits the proportion shows a tendency to reversal, while, in addition, there occurs a reduction in the rate of growth of the primary tumor, which reduction, together with the reversal in relationship, appears to be more pronounced from series to series as malignancy increases.

An attempt has been made to account for these results in the light of what has been ascertained of the modes of defense of the rabbit host against the inroads of the cells of this transplanted tumor.

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EXPERIMENTAL OBSTRUCTION OF THE JEJUNUM.

EFFECT OF SODIUM CHLORIDE INTRODUCED DIRECTLY INTO THE LUMEN OF THE INTESTINE BELOW THE POINT OF OBSTRUCTION.

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In previous communications we have pointed out the close relation of the chloride store of the blood to the toxemia of intestinal obstruction.[1–4] There is seldom clinical evidence of toxemia or a rise in the non-protein nitrogen of the blood until the chlorides reach a low level. It has been shown repeatedly, in experimental animals, that when steps are taken to maintain the blood chlorides near the normal level, a toxemia never develops even with an obstruction at so high a point as the duodenum.[2,3,5] Likewise, after the onset of toxemia, the use of chlorides therapeutically will usually save the animal unless vital organs have already been injured beyond recovery.[2,3,5]

Information thus gained experimentally, has been applied in the treatment of patients suffering from obstruction of the intestine, with excellent results.[6–8] Confirmatory clinical results have been reported by Dixon[9] and by McVicar.[10]

In our earlier experiments dealing with the therapeutic use of sodium chloride, the solutions were given subcutaneously in various

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[10] McVicar, C. S., *Am. J. Med. Sc.*, 1925, clxix, 224.

concentrations. Later intravenous injection was employed alone or combined with subcutaneous injections with gratifying results.[5] Recently we have been attempting to determine the value of sodium chloride solutions introduced directly into the lumen of the intestine, below the level of the obstruction. The results of these experiments are reported herewith.

Method.

The animals have been kept in metabolism cages. All operations were done under ether anesthesia with aseptic technique. Water was allowed *ad libitum* but no food was given during the course of the experiment. Blood for chemical analysis was obtained from the jugular vein before operation and at 24 hour intervals thereafter until the observations were ended.

The jejunum was obstructed by section about 12 inches from the pylorus by inversion of the cut ends. The obstruction was known to be complete in every instance. At the time of operation a rubber tube, for introducing the salt solution, was placed in the jejunum below the point of obstruction. The non-protein nitrogen and creatinine of the blood have been determined by the method of Folin and Wu;[11] the protein-free filtrates were made by a modification of the Folin and Wu method described by one of us;[12] the CO_2-combining power was determined by the method of Van Slyke and Cullen;[13] urea nitrogen by the Van Slyke and Cullen modification of the Marshall method;[14] uric acid by Benedict's method,[15] and the chlorides on the tungstic acid filtrate in the manner suggested by Gettler.[16]

EXPERIMENTAL.

The results of the blood studies are shown in Tables I to V. To determine the effect of water alone, four dogs with obstruction were given daily 40 cc. of distilled water per kilo of body weight directly into the jejunum below the obstruction (Table I). The animals lived from 2 to 8 days. The chemical changes in the blood in all were characteristic of the toxemia of high intestinal obstruction; *i.e.*, a fall in chlorides, a rise in the CO_2-combining power, and finally an

[11] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[12] Haden, R. L., *J. Biol. Chem.*, 1923, lvi, 469.
[13] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
[14] Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, 1914, lxii, 1558.
[15] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.
[16] Gettler, A. O., *J. Am. Med. Assn.*, 1921, lxxvii, 1652.

TABLE I.

Obstruction of the Jejunum. Given Distilled Water Directly into the Lower Part of the Jejunum below the Point of Obstruction.

Dog No.	Blood. Amount per 100 cc. Day after operation.	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
1	0	32.6	7.0	0.7	1.6	490	24.9	40 cc. dist. water per kg.
	1	36.6	4.9	0.7	1.8	430	34.3	" " "
	2	26.5	2.8	0.5	2.0	390	24.9	" " "
	3	38.5	14.0	0.8	1.4	390	24.9	" " "
	4	26.5	6.3	0.8	1.9	350	41.9	" " "
	5	26.5	5.6	1.1	1.6	360	40.0	" " "
	6		26.6			320		Died.
2	0	27.7	9.1	2.2	1.5	440	26.8	40 cc. dist. water per kg.
	1	45.6	9.8	2.2	1.6	390	21.9	" " "
	2	49.2	9.8	1.0	1.8	360	18.3	" " "
	3	60.0	14.0	1.6	1.7	310	32.4	" " "
	4	45.6	14.0	1.3	1.0	340	36.2	" " "
	5	43.6	20.3	1.2	1.4	290	24.9	" " "
	6	42.2	11.2	1.0	1.3	310	17.4	" " "
	7	40.0	11.2	1.0	1.5	360	32.4	Died.
3	0	41.6	16.8	1.7	2.2	430	25.8	40 cc. dist. water per kg.
	1	50.0	11.2	2.0	1.7	330	38.1	" " "
	2	142.0	53.2	2.2	3.6	150	32.4	" " "
								Died.
4	0	45.6	15.4	2.6	1.4	450	30.1	40 cc. dist. water per kg.
	1	48.2	9.1	2.6	1.4	430	34.3	" " "
	2	36.1	12.6	1.8	1.5	400	34.3	" " "
	3	41.6	9.1	1.9	1.3	420	30.5	" " "
	4	34.1	11.2	1.6	1.8	390	40.0	" " "
	5	42.8	6.5	1.8	1.8	370	37.2	" " "
	6	78.0	28.7	2.3	2.0	340	33.4	" " "
	7	35.3	17.5	1.3	1.5	310	28.7	" " "
	8	64.6	35.0	1.0	1.8	280	42.8	" " "
								Died.

TABLE II.

Obstruction of the Jejunum. Given 1 and 2 Per Cent Sodium Chloride Solution Directly into the Lower Part of the Jejunum below the Point of Obstruction.

Dog No.	Blood. Amount per 100 cc. Day after operation.	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
5	0	32.3	8.4	2.4	1.4	440	21.1	40 cc. 1 per cent NaCl per kg.
	1	46.8	7.7	2.2	1.7	420	26.8	" " "
	2	39.5	6.3	0.4	1.7	440	19.2	" " "
	3	34.1	9.8	1.1	1.7	410	13.6	" " "
	4	39.5	9.8	1.1	1.1	400	31.5	" " "
	5	40.0	12.6	0.7	1.2	410	45.7	" " "
	6	55.4	10.5	1.3	1.4	380	40.0	" " "
	7		11.9	1.1	1.6	350	36.2	" " "
	8	49.2	12.6	1.0	1.6	380	45.7	" " "
	9	42.2	14.7	1.5	1.8	380	37.2	" " "
	10	38.0	20.3	1.2	1.6	360	26.8	" " "
	11	38.9	9.8	1.8	1.8	370	43.8	40 cc. 2 per cent NaCl per kg.
	12	48.2	9.8	1.8	1.7	440	41.9	" " "
	13	39.5	10.5	1.6	1.5	440	34.3	" " "
	14	44.4	10.5	1.8	2.1	430	45.7	" " "
	15	31.6	9.8	1.1	1.3	370	34.3	" " "
	16	30.3	11.2	1.8	1.6	370	36.2	" " "
	17	39.5	10.0	2.2	1.4	390	47.5	" " "
	18	38.5	12.6	1.5	1.8	330	39.0	" " "
	19	31.6	7.9	2.8	1.9	380	51.3	" " "
	20	24.0	10.7	1.8	1.8	360	47.5	" " "
	21	24.6	11.2	1.4	1.5	350	46.6	" " "
	22	37.5	9.1	0.9	1.8	340	49.4	" " "
	23							" " "
	24	45.6	5.6	1.0	1.6	380	39.0	" " "
	25							" " "
	26	38.9	14.0	1.3	1.8	380	42.8	" " "
	28	34.9	11.2	0.8	1.2	370	44.7	" " "
	30	42.2	18.2	1.0	1.3	390	43.8	Died.
6	0	31.9	11.2	1.7	1.8	450	23.0	40 cc. 1 per cent NaCl per kg.
	1	35.3	7.7	2.0	1.6	400	11.0	" " "
	2	41.0	6.3	2.0	1.5	340	34.3	" " "

TABLE II—*Concluded.*

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
6	3	35.7	9.1	1.6	1.5	460	21.1	40 cc. 1 per cent NaCl per kg.
	4	57.8	8.4	1.8	1.8	460	33.4	" " "
	5	29.1	13.8	2.2	1.6	450	28.7	" " "
	6	28.0	9.1	1.5	1.5	350	24.9	" " "
	7	32.3	11.2	1.6	1.3	420	29.1	" " "
	8	37.5	10.5	1.6	1.4	420	48.5	" " "
	9	40.0	7.7	1.5	2.1	410	48.5	" " "
	10	41.6	7.5	2.3	1.6	380	34.3	" " "
	11	30.3	8.8	2.0	1.9	465	36.0	" " "
	12	42.8	16.1	1.5	1.7	380	27.7	" " "
	13	36.6	10.5		2.0	410	42.8	" " "
	14							" " "
	15	50.8	9.1	1.2	1.8	380	35.3	" " "
	16							" " "
	17	34.1	9.1	0.8	1.6	360	40.9	Developed distemper.
	18							40 cc. 1 per cent NaCl per kg.
	19	128.4	27.3			150	45.7	" " 2 " " " " "
								Died from distemper.
7	0	42.8	11.9	3.0	1.3	470		40 cc. 1 per cent NaCl per kg.
	1	34.9	10.5	2.2	1.4	430	33.4	" " "
	2	41.6	11.9	1.8	1.6	470	34.3	" " "
	3	33.0	9.8	1.9	1.4	450	22.1	" " "
	4	42.2	11.9	1.7	1.9	420	46.6	" " "
	5	48.2	10.7	2.0	2.1	390	37.2	" " "
	6	60.0	21.5	2.8	2.1	410	32.4	" " "
	7	63.2	12.6	1.7	1.6	380	30.5	" " "
	8	54.6	16.1	1.1	1.8	370	24.9	" " "
	9							" " "
	10	54.6	15.4	1.9	1.8	370	34.3	" " "
	11							" " "
	12	43.6	16.1	1.0	1.5	370	32.8	" " "
	13							" " "
	14	52.6	12.6		1.0	280	38.1	" " "
	15							" " "
	16							" " "
	17	38.0	20.3	1.4	1.1	440	41.9	Tube came away, replaced.
	18	54.6	27.9	1.4	0.9	340	40.9	40 cc. 1 per cent NaCl per kg.

TABLE III.

Obstruction of the Jejunum. Given Distilled Water Directly into the Lower Part of the Jejunum below the Point of Obstruction, after the Onset of the Toxemia.

Dog No.	Blood. Amount per 100 cc. Day after operation.	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
8	0	35.3	11.2	1.6	1.3	490	32.4	
	1	38.0	7.9	2.1	1.2	450	30.5	
	2	38.9	7.0	1.5	1.3	420	35.3	
	3	33.7	9.8	1.9	1.3	430	43.8	
	4	30.0	10.7	2.4	1.5	410	36.2	
	5	36.1	11.2	1.5	1.5	420	29.6	
	6	36.1	15.4	2.0	1.7	400	38.1	10 cc. dist. water per kg.
	7	36.6	14.7	1.9	1.5	420	53.2	No injection.
	8	39.5	12.6	1.5	1.7	390	50.4	10 cc. dist. water per kg.
	9	35.7	11.7	1.9	1.9	390	49.4	" " "
	10	32.6	9.3	1.1		380	55.8	" " "
	11	34.5	11.7	1.7	2.4	300	50.4	" " "
	12	34.5	12.1	1.8	1.8	320	43.8	" " "
	13	31.6	14.0	1.7	2.3	300	46.6	" " "
	14	107.0	51.8	1.6	2.9	260	49.4	Died.
9	0	27.3	7.5	1.6	1.6	500	25.8	
	1							
	2	33.7	8.9	1.9	2.3	420	31.5	
	3	38.9	10.3	2.1	2.6	400	33.4	
	4	40.0	11.7	2.2	2.3	390	43.8	
	5	45.6	14.0	2.7	1.6	370	46.6	
	6	45.0	16.1	4.4	2.1	350	33.4	40 cc. dist. water per kg.
	7	39.5	13.3	2.1	1.9	330	45.7	" " "
	8	31.6	14.0	1.3	2.0	300	49.4	" " "
	9	33.3	12.6	2.0	2.0	290	59.8	" " "
	10	34.1	12.6	1.2	2.0	250	54.1	" " "
	11	68.3	39.2	2.7		210	47.5	Died.
10	0	46.8	22.4	1.0	2.3	490	27.5	
	1	66.3	28.9	1.6	2.3	450	31.5	
	2	93.8	34.3	2.5	2.3	380	32.4	10 cc. dist. water per kg.
	3	143.0	73.6	3.6	2.3	310	49.4	" " "
								Died.

TABLE III—*Concluded.*

Dog No.	Blood. Amount per 100 cc. Day after operation	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
11	0	33.0	14.5	2.2	2.3	460	27.5	
	1	38.5	15.4	2.3	2.2	390	27.7	
	2	38.5	10.7	2.9	1.8	340	40.0	
	3	34.9	9.1		1.9	340	32.4	40 cc. dist. water per kg.
	4	38.9	12.6	2.3	2.3	350	43.8	" " "
	5	30.3	11.2	1.4	1.7	310	40.9	" " "
	6	35.3	10.5	1.4	1.7	310	52.2	" " "
	7	31.6	9.8	1.4	2.3	310	49.9	" " "
	8	32.6	11.2		2.4	260	49.4	" " "
	9	42.8	24.5		2.9	240	36.2	" " "
	10	131.0	55.5	2.3	3.0	240	20.2	" " "
								Died.

increase in the urea nitrogen and non-protein nitrogen. It is evident that the water alone does not influence the course of the toxemia or have any beneficial effect.

In Table II is shown the chemical findings in the blood of three dogs in which 40 cc. per kilo of 1 or 2 per cent sodium chloride solution were similarly introduced into the jejunum. One dog, in which no complications occurred, lived 30 days after the operation was performed. There were no clinical or chemical evidences of toxemia. Another animal developed distemper on the 17th day of the experiment and died 2 days later. On the day of death the chlorides were exceedingly low and the urea nitrogen and non-protein nitrogen were high. In the third dog the tube came away on the 17th day and could not be replaced. The animal died the following day.

Four dogs were given distilled water after the chlorides had fallen following obstruction and the onset of a toxemia seemed imminent. The results in these animals are shown in Table III. In one animal, in which the toxemia was slow in developing, only 10 cc. of distilled

TABLE IV.

Obstruction of the Jejunum. Given 10 Per Cent Sodium Chloride Solution Directly into the Lower Part of the Jejunum below the Point of Obstruction, after the Onset of the Toxemia.

Dog No.	Blood. Amount per 100 cc. Day after operation.	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
12	0	30.9	12.6	1.3	1.2	510	27.7	
	1	31.9	16.1	1.3	1.8	490	19.2	
	2	41.6	10.5	1.4	1.3	380	40.0	
	3	34.5	11.9	2.5	1.0	360	43.8	
	4	34.5	9.1	1.9	1.2	340	38.1	
	5	37.0	10.5	2.0	1.0	250	46.6	10 cc. 10 per cent NaCl per kg.
	6	70.6	38.2	1.9	1.2	340	44.7	" " "
	7	77.3	41.6	1.4	2.1	270	58.9	20 cc. 10 per cent NaCl per kg.
	8	41.6	17.5	1.4	1.3	360	47.5	" " "
	9	92.5	35.7	2.0	1.6	310	35.3	" " "
	10	127.0	56.0	2.2	3.0	300	48.5	" " "
	11	62.5	21.7	2.8	1.0	375	48.5	" " "
	12	32.6	16.8	1.8	1.0	370	51.3	" " "
	13	37.5	14.9	1.8	1.1	400	53.2	" " "
	14							" " "
	15	32.3	21.3	1.5	1.3	460	53.2	No injection.
	16							" "
	17	68.8	22.4	1.8	1.3	460	40.0	" "
	18	60.0	23.0	1.9	1.5	400	48.5	" "
	19	131.0	58.1	1.8		350	44.7	20 cc. 10 per cent NaCl per kg. Died.
13	0	30.9	11.2	1.1	1.5	490	28.7	
	1	31.2	12.6	1.4	1.4	490	22.1	
	2	38.9	9.1	1.6	1.3	440	18.3	
	3	27.3	4.2	1.5	0.8	430	18.3	
	4	29.4	9.8	1.6		400	34.3	
	5	25.9	13.5	1.8	1.2	470	36.2	10 cc. 10 per cent NaCl per kg.
	6		30.6	1.7	1.6	420	36.2	" " "
	7	44.4	25.7	1.3	1.4	390	38.1	" " "
	8	50.0	32.7	1.1	1.1	410	40.9	" " "
	9	42.2	23.1	1.5	1.4	430	36.2	" " "
	10	35.0	19.6	1.0	1.9	490	40.9	" " "

TABLE IV—*Concluded.*

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
13	11	36.0	21.7		1.0	630	37.2	10 cc. 10 per cent NaCl per kg.
	12	36.0	19.6	0.9	0.9	540	48.5	No injection.
	13	41.6	16.8	1.4	1.2	490	28.7	" "
	14							" "
	15	51.8	27.3	1.6	1.4	490	40.0	" "
	16	75.0	49.0	2.0	1.7	390	38.1	" "
								Died.
14	0	34.9	22.4	1.9	1.2	560	38.1	
	1	33.3	12.1	1.1	1.6	450	45.7	
	2	57.8	28.0	1.4	1.5	430	40.0	
	3	44.4	17.8	1.6	1.3	410	48.5	
	4	44.4	26.2	1.8	2.3	380	57.0	
	5	100.0	40.2	2.3	2.1	335	48.5	
	6	100.0	57.9	2.0	1.1	290	28.7	20 cc. 10 per cent NaCl per kg.
	7	55.4	30.8	1.7	1.4	330	73.0	10 " " " " " " "
	8	57.8	17.3	1.6	1.4	400	60.7	No injection.
	9	68.3	21.0	1.7	1.4	340	70.2	10 cc. 10 per cent NaCl per kg.
	10	41.0	21.5	1.2	1.6	450	66.4	No injection.
	11	50.5	33.6	1.5	1.7	350	67.3	10 cc. 10 per cent NaCl per kg.
	12	30.0	11.2	1.4	1.4	380	60.7	No injection.
	13	28.5	11.9	1.6	1.8	340	67.3	10 cc. 10 per cent NaCl per kg.
	14	25.2	9.8	1.7	1.6	400	65.5	No injection.
	15	30.0	13.1	1.3	1.9	380	69.0	" "
	16	37.0	15.9	1.8	2.3	380	68.3	10 cc. 10 per cent NaCl per kg.
	17	34.1	12.1	1.0	2.1	400	57.7	" " "
	18	33.3	14.4	1.5	2.2	460	60.5	" " "
								Died.
15	0	32.6	15.8	1.4	1.7	570	17.4	
	1	42.2	14.0	2.7	1.6	450	33.4	
	2	46.0	16.8	2.0	1.6	420	40.9	
	3	42.8	23.8	1.2	1.4	470	41.9	
	4	52.6	21.0	2.2	2.3	370	49.4	
	5	68.3	28.9	3.2	1.3	335	40.9	
	6	129.0	64.4	3.3	1.2	290	59.8	20 cc. 10 per cent NaCl per kg.
	7	111.0	68.2	3.3	1.5	440	48.5	10 " " " " " " "
	8	189.2	78.9	2.3	2.7	550	20.2	Died.

water per kilo were given. The chlorides continued to fall and the dog died on the 14th day. The other animals were given 40 cc. of distilled water per kilo. One died on the 11th day, one on the 10th, and one on the 3rd day of the experiment. All showed a gradual fall in chlorides and finally a rise in the non-protein nitrogen.

Table IV shows the findings in four dogs treated with concentrated solution of sodium chloride after the onset of a toxemia. The amount

TABLE V.

Obstruction of the Jejunum. Given 1.71 N Hydrochloric Acid Directly into the Lower Part of the Jejunum below the Point of Obstruction.

Dog No.	Blood. Amount per 100 cc. Day after operation.	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Chlorides.	CO_2-combining power.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	
16	0	25.7	9.3	2.4	1.6	500	28.7	
	1							
	2	39.5	9.3	1.3	2.6	390	36.2	
	3	120.0	55.8	2.6	2.9	360	27.5	40 cc. 1.71 N HCl per kg. Died.
17	0	36.1	11.2	1.0	1.9	480	24.0	
	1							
	2	36.1	10.3	1.8	2.4	370	34.3	
	3	34.9	14.0	2.0	2.1	360	34.3	40 cc. 1.71 N HCl per kg.
	4	62.5	26.6	1.6	2.0	330	27.5	" " " Died.

of fluid used was much less than in the control experiments with distilled water alone. Any beneficial results obtained in such an experiment must be due to the sodium chloride, since the water alone yielded no effect in the control animals. In Dog 12 the urea nitrogen had risen to 56 mg. and the non-protein nitrogen to 127 mg. after the beginning of treatment. The findings returned almost to normal, however, and remained practically so until the death of the animal, on the 19th

day. Dog 14 died on the 18th day of the experiment. Treatment was begun on the 6th day, when the urea nitrogen was 57.9 mg., the non-protein nitrogen 100 mg., and the chlorides 290 mg. The findings returned to normal and remained so until the death of the animal on the 18th day. Similarly, Dog 15 was not treated until the urea nitrogen and non-protein nitrogen had reached a very high level. The chlorides returned to normal but the urea nitrogen and non-protein nitrogen continued high until death on the 8th day. The most probable explanation here is that sufficient damage had been done to the kidney and perhaps other vital organs to cause death, even though the development of toxic bodies may have been arrested.

Since there is much to suggest that the chloride is utilized as a protective agent in the form of hydrochloric acid,[2,6] two dogs were given a hydrochloric acid solution equivalent to a 1 per cent solution of sodium chloride. The findings in these two animals are shown in Table V. It is evident, from the results, that the acid had no effect on the course of the toxemia.

DISCUSSION.

The results obtained in this series of experiments emphasize again the constancy of the changes in the blood of the dog following high intestinal obstruction. Every animal shows a fall in chlorides and many have an increased CO_2-combining power of the blood plasma. In most cases there is a rise in the urea nitrogen and non-protein nitrogen. The change in the chlorides is, however, the one constant finding.

The results in the animals treated by the introduction of sodium chloride solutions into the intestine below the point of obstruction are similar to those obtained when animals are given sodium chloride subcutaneously and intravenously. The findings are of special interest in adding further evidence of the value of sodium chloride in the treatment of the toxemia incident to intestinal obstruction.

It is evident that the sodium chloride introduced directly into the lumen of the intestine is well absorbed and as efficacious as when given subcutaneously or intravenously.

SUMMARY.

Chemical changes are reported occurring in the blood of animals with obstruction of the jejunum, in which distilled water or sodium chloride solutions were introduced directly into the lumen of the intestine below the point of obstruction.

Distilled water given daily from the beginning of the obstruction, had no influence on the development or course of the toxemia.

1 and 2 per cent salt solutions prevented a toxemia in uncomplicated cases. One animal so treated lived 30 days.

Distilled water, given after the onset of toxemia, did not alter the progress or outcome of the toxemia.

10 per cent sodium chloride solution, administered after the onset of toxemia, controlled it in most cases for a long period.

Hydrochloric acid had no effect on the course of the toxemia.

STUDIES ON UROBILIN PHYSIOLOGY AND PATHOLOGY.

III. Absorption of Pigments of Biliary Derivation from the Intestine.

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INTRODUCTION.

Evidence for an enterohepatic circulation of bile pigment has been offered in a previous communication from this laboratory.[1] It is our present purpose to record experiments on the absorption from the gastrointestinal tract, not only of bilirubin but of urobilin. Many new findings which indirectly testify to the absorption of these pigments of biliary derivation will also be described.

In the course of studies already reported,[2] dogs losing the total bile as the result of common duct intubation, and in consequence urobilin-free—the bile, feces, and urine containing none of the pigment—were fed either their own bile or urobilin-free bile from similar urobilin-free dogs. All the animals so fed exhibited a great increase of the bilirubin of the bile and urobilin appeared in it within 48 hours. From this fact and from others it became evident that urobilin appears in the normal bile only when bile pigment in some form was allowed to reach the intestinal tract. The evidence pointed to the absorption from the intestine either of the pigment or of substances out of which it might be formed. A closer study of the problem of this absorption has been undertaken. For the purpose pure bilirubin and urobilin have been used and the total bile collected from day to day in a sterile condition according to a special method.[3]

[1] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 699.
[2] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.
[3] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

PREVIOUS LITERATURE.

The majority of previous workers have assumed, on indirect evidence, that bile pigment is absorbed from the gastrointestinal tract and resecreted by the liver. The work of Wilbur and Addis[4] and of Addis[5] furthered this view while summarizing the general opinion of investigators up to that time. Recently doubt has been cast upon it by authors[6,7] who pointed out that proof of any absorption of bilirubin and urobilin is wanting,—an assertion made, be it said, prior to the publication of papers from this laboratory, which go far toward supplying such proof.[1,2] In one of the papers referred to, Broun, McMaster, and Rous[1] showed that feedings of dog bile to dogs with intubated common duct caused within the next 24 to 48 hours great increases in the bilirubin output by the liver. Furthermore feedings of sheep bile containing cholohematin were followed by the appearance of this pigment in the bile secreted in the next 24 hours. Caution was required in the interpretation of the findings with cholohematin since it has been shown that the substance is not a normal constituent of sheep or dog bile. However, the increased output of bilirubin following feedings of dog bile admit of no such objection. Recently it has been demonstrated that complete bile deprivation causes urobilin to disappear from the body, and that feedings of bile to such animals are followed by the reappearance of this pigment in the bile and feces.[2]

To these experimental findings may be added certain clinical observations of value. The crucial experiment of Friedrich Müller stands out amongst them.[8] To a patient heavily jaundiced, in whose colorless stools and dark urine there was no trace of urobilin, he fed urobilin-free pig bile. In 2 days the pigment appeared in the feces, and a day later in the urine. This experiment has since been confirmed by several workers.[9,10] Recently Meyer and Heinelt[11] injected bile directly into the intestine of human patients through a duodenal tube and found an increase in bilirubinemia, above that which is now recognized to be normal to man.

Method.

For the present study healthy dogs were used, permanently intubated, some for the collection of total bile, some for samples of bile drained from a single liver lobe only, and others with tubes so arranged that either the total bile could

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[4] Wilbur, R. L., and Addis, T., *Arch. Int. Med.*, 1914, xiii, 235.

[5] Addis, T., *Arch. Int. Med.*, 1915, xv, 413.

[6] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916–17, xlii, 264.

[7] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

[8] Müller, F., *Verhandl. Congr. inn. Med.*, 1892, xi, 118.

[9] Fromholdt, G., and Nersessoff, N., *Z. exp. Path. u. Pharmakol.*, 1912, xi, 400.

[10] Fischer, H., and Meyer-Betz, F., *Münch. med. Woch.*, 1912, lix, 799.

[11] Meyer, E. C., and Heinelt, H., *Deutsch. Arch. klin. Med.*, 1923, cxlii, 94.

be obtained in the collecting apparatus, or else it could flow back into the normal channels and so to the intestine.[2] The methods for bile collection, in 24 hour periods, and for analyses of the bile specimens have been described in previous papers,[2,12] together with the management of the animals. Only the data from animals with sterile bile will be considered in the analysis of results.

The Effect of Bile Loss on the Liver Output of Bile Pigments.

Studies of the bilirubin output into the bile were carried out upon nine dogs with the "altercursive biliary intubation,"[13] the same animals used in a previous study.[2] Following operation such an animal could be maintained either as an essentially normal dog, or as one deprived of the total bile; and the conditions could be alternated. After intubation the bile was regularly allowed to flow into the intestine for 1 or 2 weeks, that is to say until the effects of laparotomy had disappeared and the animal was in excellent condition. Previous work on animals from which all of the bile was collected had taught us that the output of bilirubin becomes practically constant within six to ten days after operation.[14] We began to collect the bile from dogs with "altercursive biliary intubation" after this condition of affairs had presumably established itself. It will be seen in the charts (Text-figs. 1 and 9 and Text-fig. 8 of the previous paper[2]) that instead of being uniform, the amount of pigment eliminated in the bile was greater in the first few 24 hour periods of collection than at any time later, and progressively declined, becoming approximately uniform only after 6 or 8 days had elapsed. The bile contained urobilin during the first 2 or 3 days of collection, but failed to show its presence thereafter (Text-fig. 9). When the bile was again allowed to enter the intestine for a week or more, and then again diverted to the collecting apparatus, the same "toboggan slide" effect in bilirubin elimination was observed.

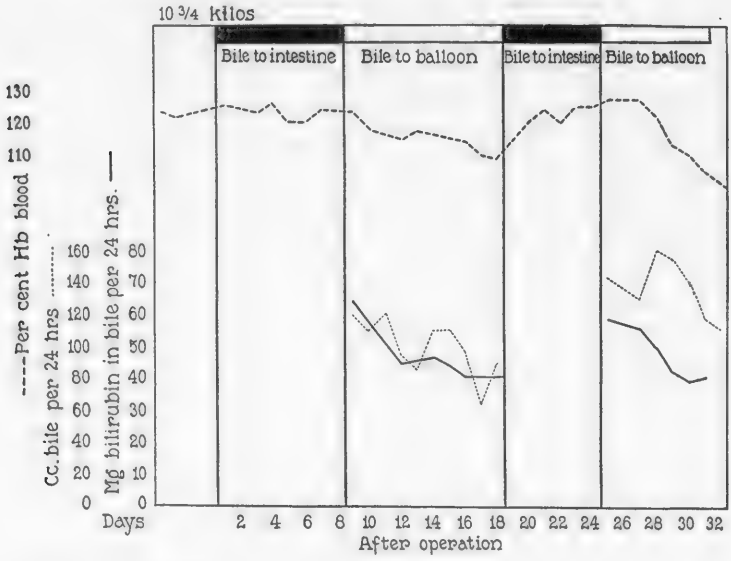
These results corroborate in a striking way our previous finding,

[12] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xli, 503.

[13] "Altercursive biliary intubation," a term coined for convenience of expression in the previous paper,[2] to describe a type of intubation of the bile ducts, by which the flow of secretion could follow one of two courses, either that to the collecting balloon, or back into the common duct and so to the intestine.

[14] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

that the occurrence of urobilin in the bile is dependent on the presence of some of this secretion in the intestine. They confirm as well the observations of Broun, McMaster, and Rous,[1] that the bilirubin falls off when bile is permanently diverted from the gut but can be increased again by bile feedings.



TEXT-FIG. 1. Effect of bile loss on the output of bile pigment.

For 8 days after operation the bile of Dog F had been allowed to flow into the intestine. When now the secretion was diverted, and collected at 24 hour intervals, the bilirubin output fell off rapidly, tending to reach a level only after the lapse of 6 days. When the bile had again been allowed to flow into the intestine, and again been diverted to the collecting apparatus, the same changes in the bilirubin output occurred. No urobilin estimations were made.

Results of Feeding Bile Containing Bilirubin and Urobilin.

The bilirubin and urobilin outputs were followed in eight dogs with intubated common duct during 10 to 30 days following intubation. At the end of this time the animals were in excellent condition, and were excreting a constant amount of bilirubin daily into the bile.

The diet was a uniform one of 1,050 gm. of bread wet with milk mixed with 450 gm. of lean meat for an animal of about 12 kilos.

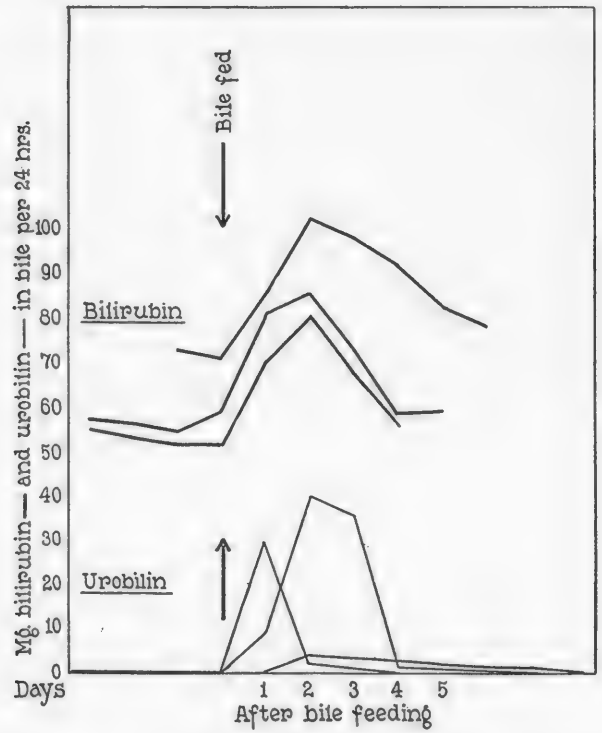
They were now fed, by gavage, 100 to 190 cc. of their own bile or that from other intubated animals—the dosage representing in each case approximately the daily output of bile pigment by the animal. This bile was urobilin-free. The amount of bilirubin administered in it was determined by the method used in quantitating the daily specimens. In each animal so fed a great increase in the bilirubin output ensued during the next 24 hours. Text-fig. 2 shows this for three animals. Prior to the feedings urobilin had not been found in the bile for many days. It appeared in each case on the day following the feeding and disappeared 1 or 2 days later. No protocols will be given, since the findings merely confirm those of the previous work.[1,2]

Far more striking are the findings when bile feedings are continued for days. Text-fig. 3 shows the sequence of events in a $16\frac{3}{4}$ kilo animal from the time of intubation of the common duct to the termination of the observations. For some days after the operation for the collection of the bile its bilirubin output decreased then reaching a fairly uniform level. The fall was greater and more prolonged than that usually seen. By the 4th day after intubation urobilin had disappeared from the bile and the stools had become acholic.

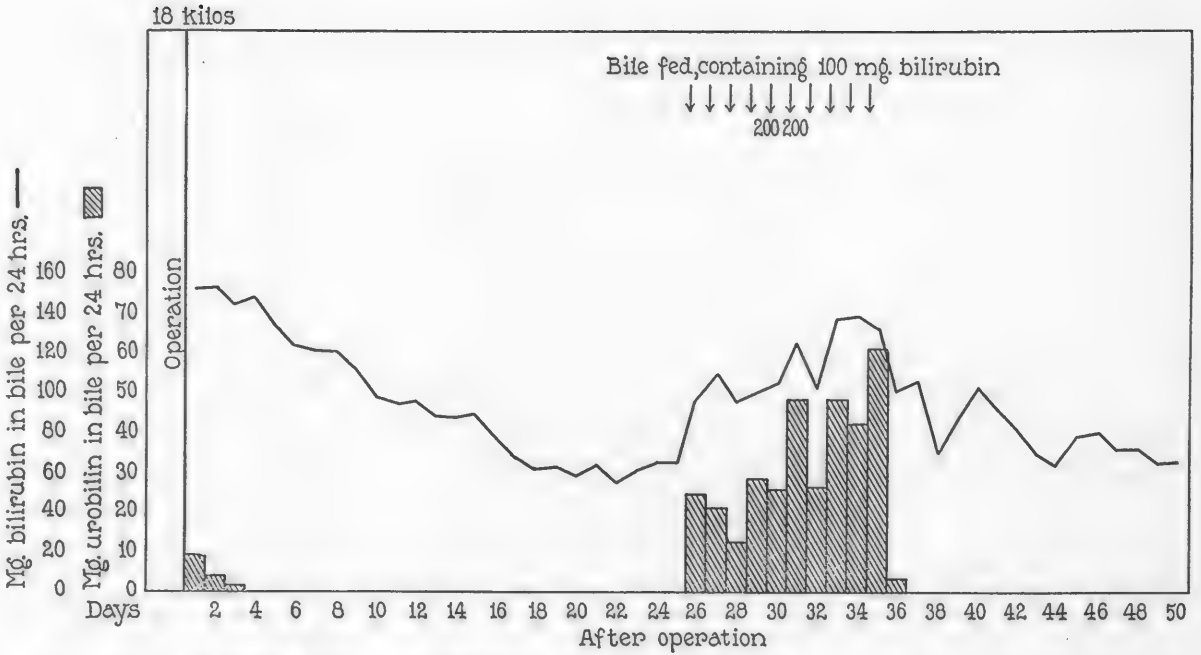
On the 25th day of the experiment, 21 days after the animal had become urobilin-free, bile feedings were commenced, by gavage, and continued for 10 days. The material used, sterile and devoid of urobilin, was derived from another intubated animal. In addition to the noteworthy increase in the bilirubin output caused by the feedings, a phenomenon sufficiently described in previous papers,[1,2] there was a reappearance of urobilin in the bile of the 24 hour period immediately after the first bile administration, and 2 days later in the feces. Both bile and feces continued to contain the pigment as long as bile was fed. Thereafter the output of bilirubin fell almost at once to its former level and the urobilin disappeared. Three of our dogs were repeatedly fed bile in this way, with similar results in each case.

In further experiments bile was given to animals intubated for the

collection of a small part of the bile only. These, too, showed an increased bilirubin elimination after feedings of bile by mouth. Text-fig. 4 shows this effect of a single bile feeding to a dog losing but 28 per cent of its bile into the collecting apparatus. The animal was in excellent condition and had been gaining weight steadily since intubation. Urobilin had been constantly present in the bile samples.



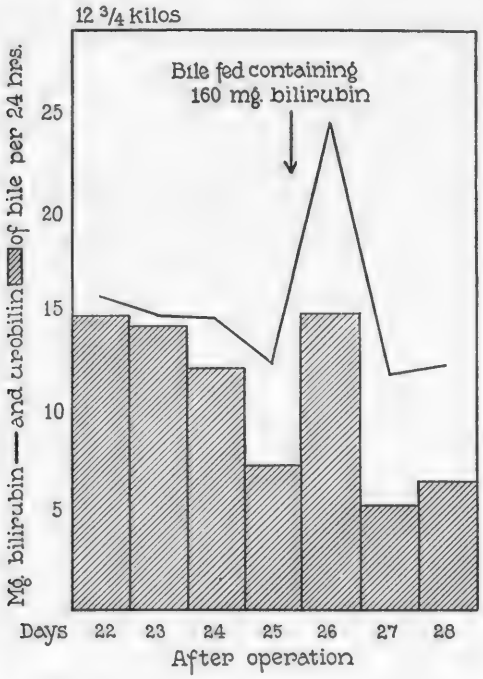
TEXT-FIG. 2. The increased output of bile pigments after a single bile feeding. The result of bile feedings to three animals with intubated common duct. The bilirubin output in each of these animals was practically uniform before the feedings were given. The bile was urobilin-free, but the pigment appeared in it promptly after the feeding and persisted there for several days. There was a synchronous rise in the bilirubin output.


18 kilos
Bile fed, containing 100 mg. bilirubin
200 200
Operation
Mg. bilirubin in bile per 24 hrs.
Mg. urobilin in bile per 24 hrs.
160 140 120 100 80 60 40 20 0
80 70 60 50 40 30 20 10 0
Days
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
After operation

TEXT-FIG. 3. The increased output of bile pigments after repeated bile feedings.

The feedings caused an enormous increase in the bilirubin output, and a reappearance of urobilin in the bile. After the feedings urobilin disappeared and the bilirubin output fell to the previous level.

Dog C, 11 1/2 kilos, male. Under ether anesthesia cannulation of the duct to the left lateral lobe of the liver only. This lobe was determined at autopsy to be 28 per cent of the total liver. The animal was in excellent condition, on the standard mixed diet.



TEXT-FIG. 4. The increased output of bilirubin after feeding bile to an animal deprived of only a small part of the secretion.

It will be seen that on the day after a bile feeding involving 160 mg. of bilirubin there was an increase of more than 75 per cent in the output of this pigment. 2 days later the output had dropped again to the previous level.

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26 days later the animal had gained 1 kilo in weight and was putting out sterile bile with a quite uniform bilirubin content, having 14.7, 14.75, and 12.18 mg. of bilirubin daily, from the 24th to the 25th days, in the 28 per cent sample of the secretion, during each 24 hour period. As in all animals so intubated, the bile and stools contained urobilin. On the 26th day 190 cc. of sterile urobilin-free bile, containing 160 mg. of bilirubin, derived from a dog with intubated common

duct, was fed by mouth. As Text-fig. 4 shows a rise in the bilirubin elimination in the bile sample from 12.18 to 24.25 mg. occurred in the following 24 hours; with a fall in the next period to the usual level,—12.1 mg.

It might be argued that the increased bile pigment output after bile feedings in animals losing all of their bile might be due to a betterment in digestive or other conditions. But this explanation can scarcely be invoked in instances such as the foregoing. There were three such in two different animals.

The Effect of Feeding Bile Salts.

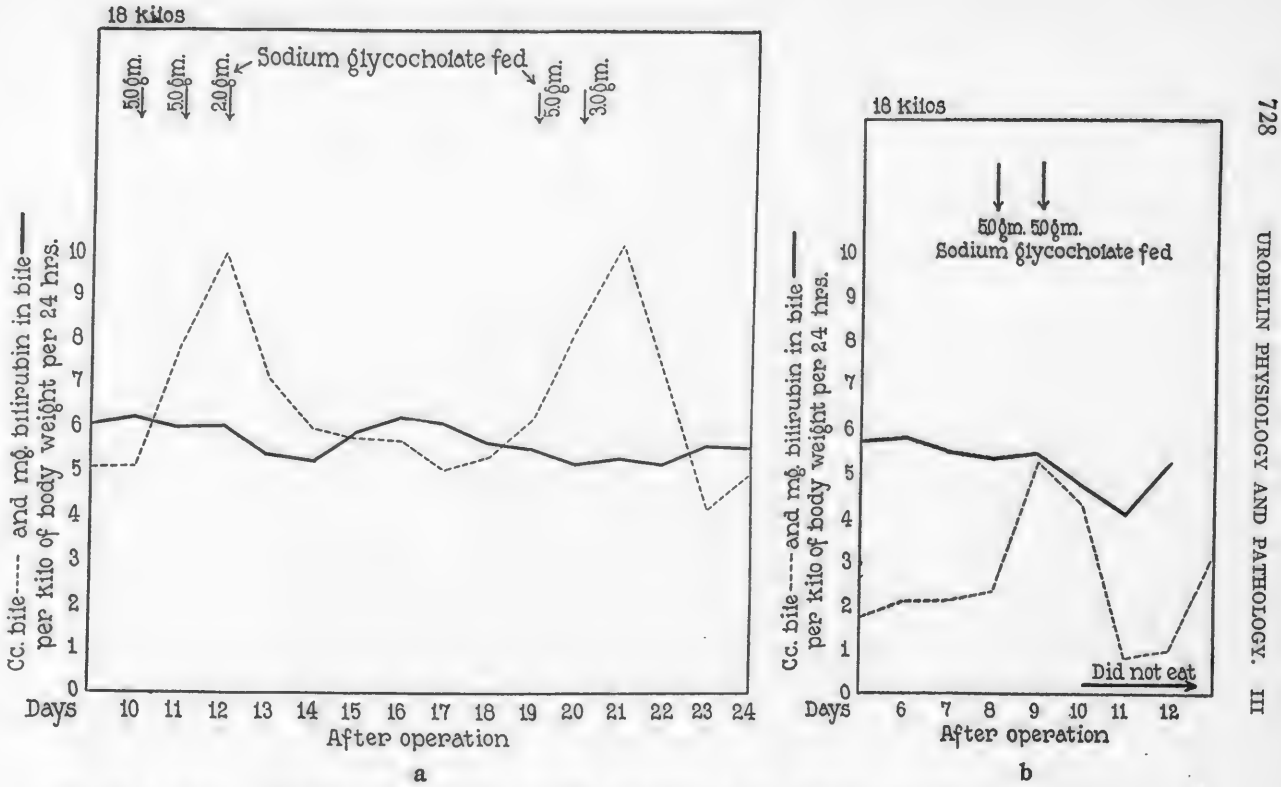
Feeding of whole bile to intubated dogs brings about not only an increase in the bilirubin output, but a great one in the quantity of bile, owing, of course, to the cholagogue action of the bile salts. When these latter fail to reach the intestine, as during bile collection by the "altercursive" method, the bile volume (Text-fig. 1) undergoes a gradual decrease. The increase in bilirubin eliminated after bile feeding is not proportional to the greater volume of bile put out. That bile volume and pigment quantity bear no relation to each other has already been shown.[1,15]

It seemed important to test the possibility whether the giving of bile salts by mouth might not induce the observed increase in bilirubin by a destruction of red cells. For bile salts exert a hemolytic action *in vitro*, though only to a marked degree when serum is absent. Accordingly, four dogs with intubated common duct, and two with a "sampling intubation," all with a uniform daily output of bilirubin, were given varying amounts of sodium glycocholate by gavage. The same ration as previously was also provided. The outcome of the experiment, in two typical instances, is shown in Text-fig. 5, *a* and *b*. The bile salts produced a strong cholagogue effect without altering the bilirubin output of the 24 hour sample.

The Preparation of Purified Bile Pigments.

It is evident that one cannot ascribe the increased output of bilirubin after feedings of whole bile to the action of the bile salts ingested

[15] McMaster, P. D., *J. Exp. Med.*, 1924, xl, 25.


18 Kilos
50 gm.
50 gm.
20 gm.
Sodium glycocholate fed
50 gm.
30 gm.
Cc. bile---- and mg. bilirubin in bile——
per kilo of body weight per 24 hrs.
0 1 2 3 4 5 6 7 8 9 10
Days 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
After operation
a
18 Kilos
50 gm. 50 gm.
Sodium glycocholate fed
Cc. bile---- and mg. bilirubin in bile——
per kilo of body weight per 24 hrs.
0 1 2 3 4 5 6 7 8 9 10
Did not eat
Days 6 7 8 9 10 11 12
After operation
b

TEXT-FIG. 5, *a* and *b*. Bile salts by mouth act to increase the amount of bile but not of bilirubin.

Repeated doses of the bile salts were given to the dogs by mouth. Dog B received 5 gm. of sodium glycocholate on 2 successive days, and on the 3rd day a 2 gm. portion. A week later two more 5 gm. doses were given. Dog E received, on 2 successive days, 5 gm. doses. The charts show that the strong cholagogue effect of the feedings was unattended by alteration in the bilirubin output.

therewith. Like the large initial bilirubin output in animals with an "altercursive intubation," and like the relatively great output of animals with a mere "sampling intubation," the phenomenon must be due to some other constituent of the bile reaching the intestine. The explanation that this constituent is the bile pigment itself, which is in part reabsorbed, and resecreted, would, in view of present knowledge, seem the rational one. We have proceeded to test it.

Purified bilirubin and urobilin, were prepared and administered to intubated animals.

Preparation of Urobilin.—The method of preparation of urobilin from dog feces has already been described in another paper.[12]

Preparation of Pure Bilirubin from Dog Bile.—In all, about 10 liters of dog bile from the collecting balloons of animals with intubated common duct were utilized. As fast as a few 100 cc. had been assembled on ice it was used. Each lot was precipitated within 2 days so that little or no transformation of bilirubin to biliverdin would take place in it.

To each 100 cc. of fresh bile used, 10 cc. of 20 per cent NaOH was added and the mixture was brought to a boil. To the hot mixture, glacial acetic acid was added until precipitation was complete; 10 to 12 cc. usually giving the best results. Two precautions were found necessary for successful extraction of bilirubin, the use of fresh bile only, containing but little biliverdin, and boiling in a closely covered container, under circumstances making for the exclusion of air. The precipitate was collected on a filter paper and washed with water until the filtrate appeared clear and proved acid-free. Precipitate and filter paper were next removed to an extraction thimble in a Soxhlet apparatus, and extracted with alcohol until the latter came away clear. The alcohol was then replaced by chloroform, and the extraction repeated, the chloroform extract filtered, the excess of chloroform distilled off and recovered, and the remaining filtrate evaporated to dryness in a current of air. This dry residue was washed again with alcohol, the fluid poured off, washing done with ether, which was in turn poured off; and washings with alcohol and with ether alternated as long as anything came away. The residue was then taken up in chloroform again, filtered, and the process of purifying repeated until only crystalline bilirubin remained.

Neither the bilirubin nor the urobilin showed any traces of bile salts by Hay's or Pettenkofer's test. The color intensity of solutions of the bilirubin, on the addition of acid alcohol, proved to be slightly stronger than that given by a Schuchardt preparation from ox bile. For the comparison a Duboscq colorimeter was used.

The Effects of Feeding Purified Bilirubin.

Pure bilirubin was given to eight dogs, three with intubated common duct, one with a "sampling intubation," and four with the "altercursive intubation."

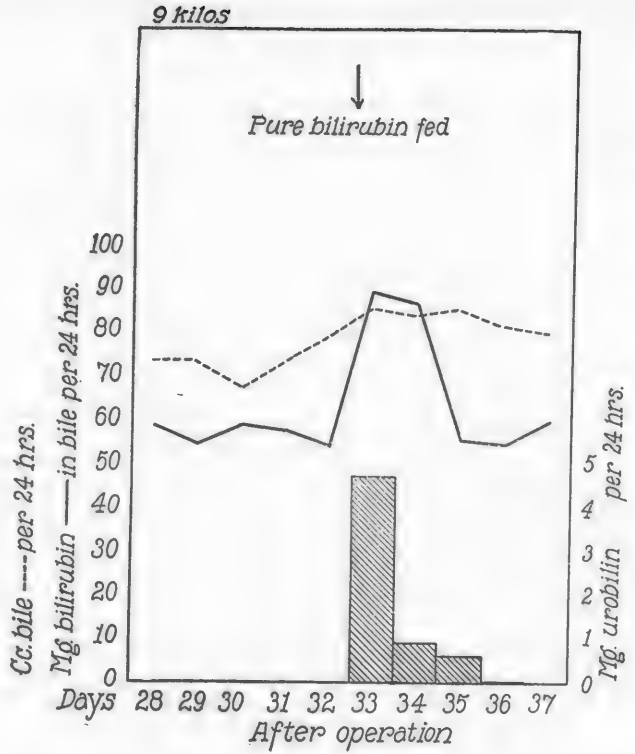
In general, 100 mg. were given, approximately the amount lost daily in the bile by most of the animals. It was dissolved before administration by the addition to each 100 mg. of 30.0 cc. of distilled water containing 1 cc. of 20 per cent NaOH. Once in solution more water could be added without any falling out of pigment, and the volume was thus made up to that of the daily secretion of bile of the animal to receive the dose. Colorimetric determinations of the bilirubin, made by the same method as the quantitations of the daily bile samples, were found to check closely with the values obtained gravimetrically. Just before the solution was given it was rendered neutral or very faintly acid by the addition of $\frac{N}{10}$ hydrochloric acid, not enough being added to produce a change from bilirubin to biliverdin. To the animals with a common duct or a "sampling intubation" the solution was fed in divided doses, several hours apart, by stomach tube. It was injected in divided doses through the efferent tube into the common duct of those with the "altercursive intubation."

In every case the result was the same; great increases in the bilirubin output in the bile followed the feedings. Text-figs. 6 and 7 illustrate the results of bilirubin feedings by mouth to animals losing all of the bile. It will be seen that immediately after a single feeding of bilirubin the output of this pigment underwent an abrupt increase and did not return to the previous amount for several days. At the maximum there was a 65 per cent increase in the 24 hour specimen (see Text-fig. 6). Urobilin reappeared in the bile following the bilirubin gavage and disappeared again only after the course of several days. With all this there was no marked change in the bile quantity. Therein the findings differ notably from those obtained by whole bile feedings, in which the cholagogue action of bile salts is apparent.

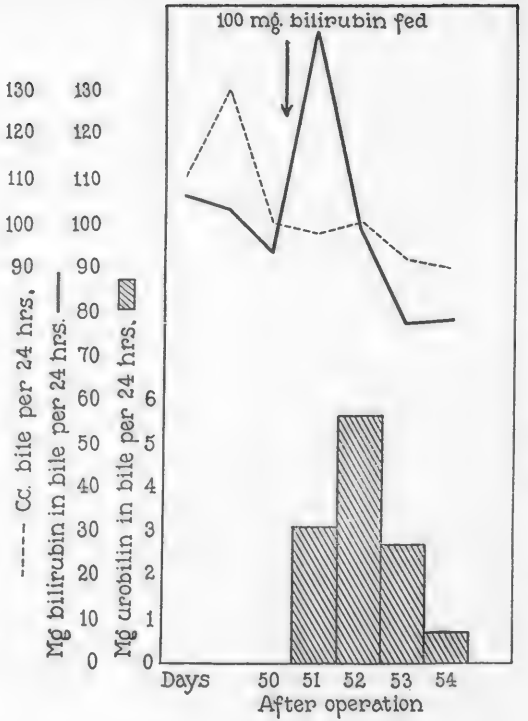
Like findings were obtained in Dog C, intubated for the collection of bile samples only and losing 28 per cent of the total secretion in this way, while the remainder passed as usual into the intestine (Text-fig. 8).

Effects of the Introduction of Purified Bilirubin into the Duodenum.

In the foregoing experiments the possibility of a direct gastric absorption of pigment, or of a gastric influence upon the bilirubin solution, rendering it capable of absorption by the intestine, could not be ruled out. To test these possibilities bilirubin in solution was instilled directly into the duodenum of dogs through the efferent



TEXT-FIG. 6.

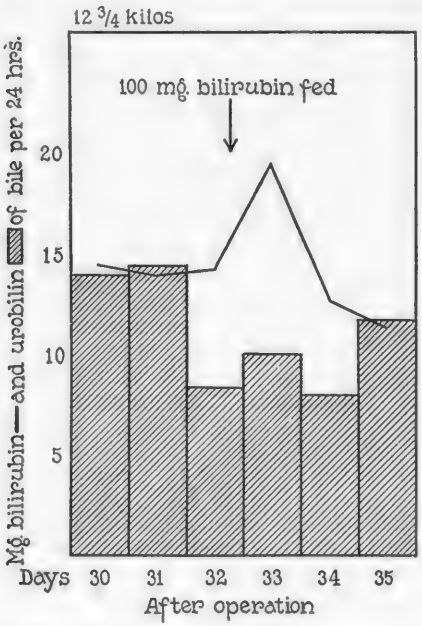


TEXT-FIG. 7.

TEXT-FIGS. 6 and 7. Effects of feeding purified bilirubin to animals losing all of the bile.

Dogs A and B. At the points indicated by the arrows pure bilirubin solution was fed by mouth. Previous to this the bile had been urobilin-free, and the daily output of bilirubin had been constant. Following the feeding urobilin appeared in the bile, and there was a noticeable temporary increase in the content of bilirubin. The bile quantity did not change.

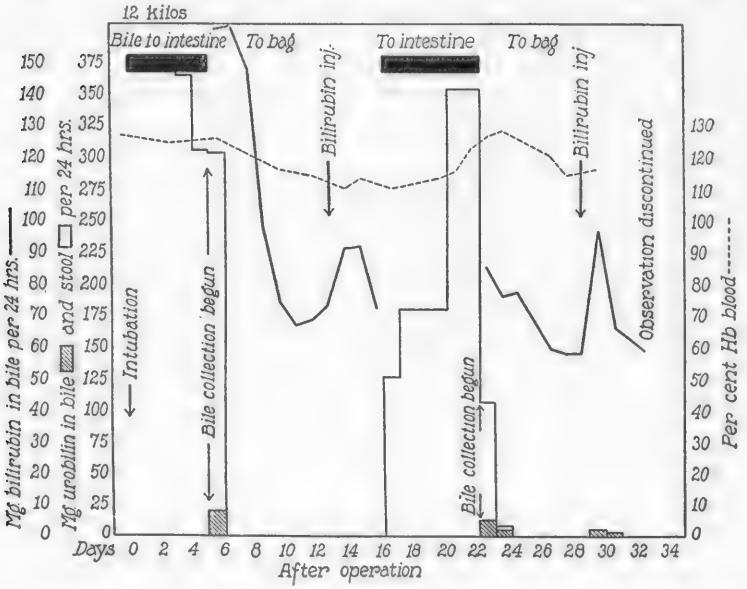
tube of the "altercursive intubation" apparatus, which is the one that connects with a cannula inserted into the lower portion of the common duct.[2] Both before and after the injection the total hepatic bile was collected as usual through the afferent tube, which connected another cannula, inserted into the upper part of the common duct, with the collecting balloon. Text-fig. 9 depicts the findings in an animal to which bilirubin was adminstered at widely separate intervals. A brief description of the experiment follows.



TEXT-FIG. 8. Effect of feeding purified bilirubin to an animal with a "sampling intubation."

The actual alterations observed in the bilirubin output in the bile sample have been charted, not the calculated changes in total output, which were much more considerable. It will be seen that the feeding of pigment was responsible for a notable increase in the output of it. Urobilin was present in the sample bile throughout the period of observation as would follow from the circumstances of the case. The observed variations in the amount of this pigment fell within the ordinary limits.

Dog D, male, 12 kilos. During the first 5 days after intubation the bile was allowed to flow into the intestine. The amount of urobilin in the stools of this period was considerable. It fell off rapidly, though, and disappeared after bile collection had been begun. In the bile first collected there was far more bilirubin than later, and the day-to-day quantitation yielded the usual "toboggan slide" curve. When a level appeared to have been reached, the content of the bile being 66.6, 67.5, and 72.3 mg. for three successive 24 hour periods, 100 mg. of



TEXT-FIG. 9. Effect of introducing pure bilirubin into the duodenum of an animal with an "altercursive intubation."

A notable rise in the bilirubin output followed the injections, on both the occasions upon which they were practiced. The other findings are discussed in the text.

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the solution of purified bilirubin was given in divided portions through the efferent tube into the duodenum. This was on the 13th day. The animal's bile of the 14th day contained 90.0 mg. of bilirubin and that of the next 24 hour period 90.6, with a return to 71.2 mg., a normal figure, 24 hours later. No urobilin appeared in the bile following the bilirubin administration. The finding was unique in this respect. Bile collection was now stopped, and the secretion allowed to reach the intestine as previously. After an interval of 1 week the bile was again

diverted to the collecting balloon, and in due course the experiment was repeated, using 100 mg. of pure bilirubin. For the 3 days prior to the administration of the pigment the bile had contained 58.9, 57.4, and 57.7 mg. of bilirubin in each 24 hours. The amount on the day following the injections rose to 95.0 mg. of bilirubin, an increase of 65 per cent. It fell in the next 24 hours to 65.2 mg., almost a normal value, and 2 days later it approximated this closely. Urobilin appeared in the bile following the pigment administration. It had been found too for a brief period immediately after bile collection was begun, just as previously.

Similar findings were obtained in two other animals, that is to say, in these there was not only a rise in the bilirubin output in the bile but a reappearance of urobilin in it as well, after each injection of pure bilirubin into the duodenum.

The Effect of Urobilin Feedings.

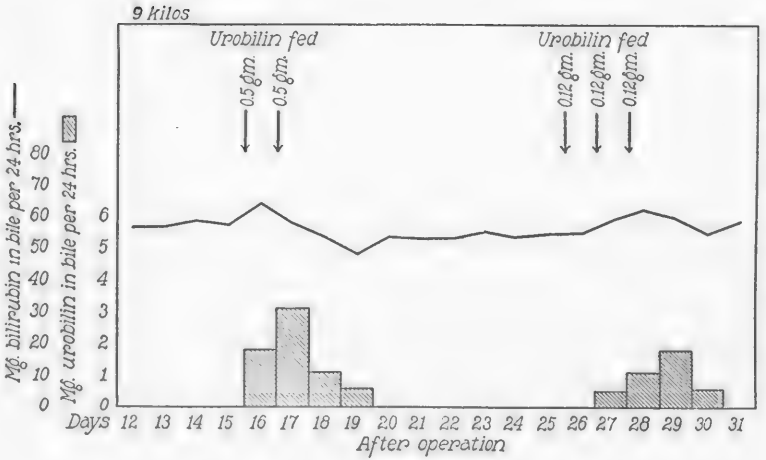
The feeding experiments were repeated with purified urobilin. Evidence had been obtained in our previous work[2] that this pigment, as well as bilirubin, is absorbed. The findings were not as striking as those with bilirubin, as was to have been expected since much of the pigment is probably reduced in the intestine to unrecognizable forms. The results, however, were definite enough to demonstrate an absorption of it clearly.

For administration by stomach tube 100 to 500 mg. of the purified pigment were taken up in 50 cc. of 1 per cent ammonium hydroxide and neutralized. Six such experiments in all were performed, two with each of three different dogs, intubated for total bile deprivation. The bile, feces, and urine of these dogs were free from urobilin before the feedings. Each animal, on the day after, showed urobilin in the bile and at times continued to show it for another 48 hours. In some instances there was a slight increase in the bilirubin output from the liver after the feedings, suggesting that perhaps part of the resorbed urobilin was converted to bilirubin.

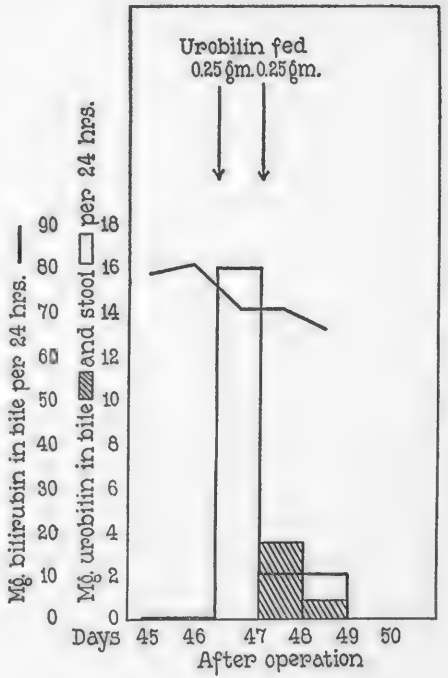
In one instance a $16\frac{3}{4}$ kilo dog, fed 250 mg. of the pigment on 2 successive days, urobilin appeared not only in the bile and stool but in the urine as well, and the bilirubin of the bile was increased. Since the findings were artificial they have not been charted.

Specimen protocols follow (Text-figs. 10 and 11).

Dog A (Text-fig. 10), 9 kilos. Cannulation of the common duct was accomplished as usual under ether anesthesia. The animal remained in excellent condi-



TEXT-FIG. 10.



TEXT-FIG. 11.

TEXT-FIGS. 10 and 11. Results of feeding urobilin to dogs with intubated common duct.

The bile was urobilin-free prior to the pigment feedings, and the output of bilirubin was uniform. At the points indicated by arrows the pure pigment urobilin was fed. In the bile of the following 24 hours urobilin made its appearance, persisting for 1 and 2 days after the last feeding. The slight increases in bilirubin in Text-fig. 10, are too small to allow of definite conclusions. Note the absence of urobilin from the bile save after the feedings.

tion and was kept on the standard diet of bread, milk, and lean meat. For 2 weeks no urobilin was present in bile, feces, or urine, and the output of bilirubin in the bile became uniform after the usual postoperative changes. On the 15th day 0.5 gm. of urobilin in solution was fed by mouth.

The hepatic bile of the 24 hours following the feeding contained much urobilin and there occurred a slight increase in the bile pigment. Another feeding of 0.5 gm. of urobilin was given with result that it doubled in quantity in the bile. A week later, when the bile had again become urobilin-free, the pigment was again given by mouth in weak alkaline, aqueous solution, on several successive days, to the amount each time of 120 mg. The pigment promptly reappeared in the bile and persisted there for 3 days after the feedings had been stopped.

Dog E (Text-fig. 11), 16¾ kilos. Common duct intubation as usual under ether anesthesia. During 46 days thereafter the animal was in excellent condition and no urobilin was present in bile, feces, or urine. 250 mg. of urobilin were now given in neutralized ammoniacal solution by mouth. The following day urobilin appeared in the urine and stool but not in the bile. Another feeding of it was given, and for 2 days thereafter the bile was found to contain it to the amounts of 4.0 and 1.0 mg. respectively in each 24 hour period.

The results in the third animal will not be charted since they were in all ways similar to those described.

The changes, though not as pronounced as those occurring on bilirubin administration, show that feedings of pure urobilin to intubated dogs yielding bile free from the pigment, result in its presence in the secretion.

DISCUSSION.

The experiments here reported supply direct proof that both bilirubin and urobilin are absorbed from the intestine. In previous papers from this laboratory it was shown that, after bile feedings, much more bilirubin is excreted into the bile[1,2] but the possibility that the increase is due to hemolysis from absorbed bile salts could not be wholly ruled out. This has now been accomplished. Feedings of sodium glycocholate as such, even in large amounts, cause no change in the bilirubin output, though greatly increasing the bile quantity. In one of our previous papers[2] it has been shown that bile feedings cause an increased bilirubin and urobilin output by animals with a "sampling intubation," and when they are given to animals losing all of the secretion into a collecting balloon cause the latter pigment to reappear in the bile so obtained as well as an in-

creased bilirubin yield. It has remained to determine whether feedings of bilirubin by mouth, or direct introduction of it into the duodenum, bring about the same effects or further whether they follow upon feedings of purified urobilin.

This has now been done. The findings leave no alternative to the conclusion that an enterohepatic circulation of bile pigments exists under normal circumstances. That this is true of bile salts has long been known, and that it holds true for cholesterol has recently been shown in this laboratory.[15] Bile is treated by the organism as though it were anything but a waste product. But can one assume that the physiological mechanism for the conservation and reutilization of its major ingredients is purposeful in character? The bile salts have well known uses; and evidence has been presented[16] that bilirubin is conserved in porportion to the need for hemoglobin from which it is primarily derived. The amount of cholesterol put out in the bile, which might, at the worst, be lost therewith, is negligible; and the absorption of it that takes place from the gut may be merely one instance of a general phenomenon. One can only conjecture as concerns a possible use of urobilin.

The fact should be emphasized here that our observations were made upon healthy, robust animals. In succeeding papers the findings after liver injury and blood destruction will be described. The enterohepatic circulation of bilirubin and urobilin is of special practical interest in such connection.

The phenomenon of pigment reabsorption, that we have demonstrated, would appear to be far reaching in its implications. It must be taken into account in any hypothesis of pigment metabolism. The clinical utilization of the urobilin output in the feces as a measure of blood destruction should only be undertaken with the understanding that urobilin is subject, not only to degradation but to reabsorption.

SUMMARY.

Direct proof of the absorption of bile pigments from the intestinal tract is presented.

[16] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 733.

Feedings of pure bilirubin by mouth to dogs, like those of whole bile, increase greatly the amount of this pigment in the hepatic bile. Control feedings of bile salts, while greatly increasing the bile quantity, cause no change in the pigment output. Instillations of pure bilirubin directly into the duodenum of dogs, appropriately intubated, are followed by pronounced increases in the bilirubin output in the bile.

Feedings of pure urobilin to dogs, yielding urobilin-free bile, as result of diversion of the secretion from the intestine, are followed by the appearance of this pigment in the bile.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

IV. Indicated Differences in the Reaction of the Organs on Vital Staining with Phthaleins.

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The color phenomena in mice vitally stained with phthaleins of slightly alkaline range are such as would point to the existence of noteworthy differences between the reaction of the blood and tissues.[1] They are indicative, furthermore, of characteristic differences in the reaction of some, at least, of the organs. The results of a further investigation, carried out with phthaleins of frankly acid range, will here be set forth, and the findings as a whole will be discussed.

The technique of vital staining with the phthaleins, and the precautions essential to proper examination of the tissues, have already been described.[1] In animals stained with cresol red and phenol red, it was found that the hue of blood plasma from the right heart corresponded with that manifested at about pH 7.38 in buffer solutions, whereas the most alkaline of the tissues, judging from its color, the connective tissue, appeared to have a pH of 7.2 or slightly less, the tendons seeming nearly, but not quite, so alkaline. The other stained tissues had without exception colors indicating that the reaction lay beyond the ranges of the indicators on the acid side, that is to say at or beyond pH 6.6.

Brom Thymol Blue.

The indicator which best covers the range immediately beyond phenol red on the acid side is, under ordinary circumstances, brom thymol blue, which is blue at pH 7.4, and passes, with progressive increases in hydrogen ion concentration, through green to a clear yellow at pH 6.0. Unfortunately it is toxic for animals.

[1] Rous, P., *Science*, 1924, lx, 363; *J. Exp. Med.*, 1925, xli, 451.

When agar, or an open glass tube containing the dye, is introduced beneath the skin of rats, there soon follows a widespread blue edema, and the lymph from the region is blue for a short time after the swelling has subsided,—sufficient evidence that the reaction of the fluids is alkaline, as would be expected. Immediately about the glass or agar macrophages can be found with granules colored bright yellow by the indicator, like those of similar elements accumulating in the abdominal cavity of mice after an intraperitoneal injection of it.[2] The agar itself is blue. The findings are wholly different when a generalized staining had been produced, in mice, by intraperitoneal injection of a 1 per cent solution of the dye. Under such circumstances indications of acidosis are seen almost from the first, in a yellowing of the hairless surfaces; and after 2 or 3 hours these have a pronounced greenish yellow hue. The animals are by this time prostrated. On dissection under ether the tissues in general are intensely yellow, and the fluid recovered from the peritoneal cavity is likewise yellow, not blue like the solution originally introduced. The hue is such as would indicate under controlled conditions a pH at least as low as 6.2. That the phthalein has retained its indicator characteristics is proven by the application of weak alkali which at once turns the stained tissue a sharp blue. The abnormal character of the general acidosis is attested not alone by all previous knowledge of body conditions and by the local blue coloration when the indicator is introduced into the subcutis, but by the findings with brom cresol purple to be described further on. The urine and feces are colored yellow by the indicator excreted with them.

The change toward alkalinity which regularly takes place in subcutaneous tissue exposed to the air[1] can be followed on the everted skin flaps of mice stained with brom thymol blue. A very gradual alteration in hue from yellow through green to blue takes place.

Brom Cresol Purple.

Brom cresol purple,—which is an outspoken purple at pH 6.4 to 6.6, under controlled conditions, and orange yellow at pH 5.4,—diffuses so rapidly that when introduced beneath the skin as a solid within agar or glass it rapidly colors the whole animal pale purple, and is almost as rapidly excreted, with result that only a fleeting local coloration is obtained. The amount required to effect pronounced staining after an intraperitoneal injection comes close to that which is lethal, but by careful dosage such staining can be effected without symptoms of illness. 1 cc. of a 1.7 per cent solution of the sodium salt of brom cresol purple will render a 30 gm. mouse deep purple within half an hour; but by the next day the animal is un-

[2] Rous, P., *J. Exp. Med.*, 1925, xli, 399.

stained, so swiftly is the indicator removed through the kidneys and liver; and no ill consequences develop.

When the sodium salt of the indicator is prepared by a gradual addition to it of $\frac{N}{20}$ sodium hydroxide, there often results a chemical change profoundly affecting the color range, and rendering the specimen useless.[3] Mice injected with the altered material stain a dirty green, not purple, and the hue of many of the organs is anomalous. The change can be avoided if the phthalein is suspended in a little salt solution, and the hydroxide in $\frac{N}{1}$ to $\frac{N}{5}$ solution is added all at once. Tests in graded buffer solutions must be made to determine whether the material to be injected has the characteristic color range.

The staining is at a maximum about 1 hour after an intraperitoneal injection. *The hairless surfaces* then appear vivid purple; but when the ear is pressed between glass slides to free it of blood it is found purply blue by transmitted light. Very little of the indicator is voided into the urine but a great deal is present in the plum colored feces.

The blood plasma and the *lymph* from the interior of glands, like the *fluid of subcutaneous regions* into which the indicator has been introduced in an agar button or a glass tube, are intensely purple. The *red cells* appear purply red when viewed in thick layer. The *leucocytes* fail to stain. Agglutinated *platelets* appear sky blue.

The matrix of the *cartilage* of ear, tail, and sternum is a brilliant purplish blue. Well vascularized *bone* stains deeply, in a slightly different blue, but dense lamellar tissue is scarcely tinted, when at all. The cartilage cells appear unstained, whereas the osteoclasts are outlined in pale blue as if by indicator on the cell surface. In the half grown animal beautiful color gradations can be made out in the region of the joints, and the skull sutures, the bone appearing deep azure, the marrow red-purple, the osteoid tissue an intense dark blue, and the cartilages a lighter, purply one.

The *tendons* are diffusely blue.

The lymph nodes appear purple to the unaided eye, as is well seen in the case of inguinal glands viewed *in situ*. But this hue is due to an intense staining of the lymph, which prevails, as in the case of phenol red, over the relatively slight coloration of the tissues. When a gland is cut open and the fluid expressed from it by gentle pressure the lymphoid tissue is seen to be everywhere of a pale greenish yellow, and the connective tissue scaffolding and capsule a contrasting purple. If weak alkali is brought in contact with the lymphoid tissue, it likewise becomes purple. The horny portion of the *epidermis* is not stained but the basal layer is purply blue.

[3] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1923.

The connective tissue is pronouncedly stained, as by all the phthaleins. It is a vivid purple. The staining is mainly, if not entirely, in the interstitial tissue. Whether the cells become colored is not certain.

The nerves and *brain* show no tinting, but the nerve sheaths are blue to purple, as would follow, doubtless, from their character as connective tissue.

The adipose tissue is a greenish yellow in the gross, with far more of yellow than is normal to it. Under the microscope those portions of it containing large and regular fat globules, the stable fat depots, that is to say, appear either unstained or faintly blue or purple; but where the cells contain many small fat globules there is a distinct greenish yellow suffusion. The fat itself is uncolored. The vessels contain purply blood, as everywhere else; and the supporting tissue is blue to purple. The application of $\frac{N}{20}$ NaOH causes the greenish yellow regions to become purple.

Bits of *voluntary muscle*, removed and inspected as rapidly as possible, appear light green to yellow, according to the proportion of purple lymph and blood associated with the yellow fibers. The latter appear only slightly yellower than normal but when the cut ends are brought in contact with $\frac{N}{10}$ NaOH the hue of the muscle substance changes to an even purple as the alkali penetrates lengthways along the fibers.

The spleen: The blood content causes this organ to appear darkly purple, but flattened bits show trabeculæ that are blue, and Malpighian corpuscles of a pale yellow-green. The elements of the splenic pulp are not perceptibly stained.

The pancreas has a diffusely yellow-green parenchyma. Its vessels have blue walls. The fluid expressed from the organ on gentle pressure is purple.

The kidneys: Relatively little of the indicator is excreted into the urine, as already stated, yet the renal cortex is, in most instances, intensely colored, usually yellow, in contrast with the medulla which is blue. Exceptionally the cortex is purple, or green with some blue tubules, and then the medulla is yellow. Always the glomeruli appear pale purple by reason of their content of stained blood.

The bile in the *gall bladder* is often so purple as to appear nearly black; but sometimes it is reddish yellow, becoming purple only after alkali has been added. The viscus can be inspected as a whole by seizing it at the neck with forceps and evulsing it, with contents undisturbed. It is then pressed between mica surfaces to force out the bile, and viewed forthwith by transmitted light. The wall of the bladder is then seen to be yellow to green.

The liver bile, as viewed in the larger intrahepatic ducts, appears purple microscopically. The cells of the hepatic parenchyma round about are pronouncedly yellow-green, becoming an intense purple with alkali. Even under the highest magnification the yellow-green staining appears diffuse. Intracellular fat globules have merely the normal hue. Glisson's capsule appears blue, and there is no distinctive staining of the Kupffer cells.

The suprarenal bodies have a greenish yellow medulla and a cortex which appears unstained.

Pieces of the *heart muscles* removed from the organ while it still beats show a staining less in degree but otherwise similar to that in the voluntary muscles.

The lung tissue is a pale greenish yellow, when viewed under the microscope.

Needless to say, the same precautions were observed in the routine examination of stained tissues,—oil bath, mica slides and covers, etc.,—as in the case of the indicators previously employed.

To obtain the foregoing data many animals were stained and examined; and in some only a few organs were inspected and this hurriedly after the etherization, in order to avoid so far as possible the complication of acidosis incident thereto. In the case of some indicators previously studied, of a comparatively alkaline range,—cresol red and phenol red,—this acidosis caused a change in the general coloration of the animal, through a change in the reaction and color of the blood; but no associated alteration could be made out in the hue of the organs. With brom cresol purple such indications of an ether acidosis are not met with, nor, in view of the range of the indicator, were they to have been expected. Asphyxia, on the other hand, induces a definite change in the color of the hairless surfaces, from purple to purply green. This can be seen during life in animals breathing infrequently after the injection of an overdose of the indicator. Companion animals with a more normal respiration, fail to show the change. When a skin flap of an anesthetized mouse is everted and exposed to air, the purple of the subcutaneous tissue rapidly intensifies as it becomes more alkaline through local ventilation; and if the mouse be placed under the funnel apparatus described in a previous paper,[1] and the flap exposed to carbon dioxide, its purple soon passes into the yellow-green of a local acidosis.

In work with brom cresol purple there is an unavoidable complication, namely dichromatism.

Many manifestations of this phenomenon are encountered in the tissues. Not a few stain blue or purply blue instead of purple; while others have a green or yellow-green hue, such as is seen in buffer solutions only under special optical conditions. All the anomalous colors can readily be duplicated *in vitro*, as will be shown in a later paper. That the blue is associated with alkalinity is proven by its failure to alter to purple when the tissue is brought in contact with alkali; and by its prompt change to yellow when acid is employed instead. The green

hue can be seen in buffer solutions of the indicator at a pH close to the middle of the color range when these are viewed by a mixture of transmitted and reflected light. It changes promptly to yellow with acid, and to purple with alkali. That it is not a manifestation of some peculiarity in the constitution of the cells wherein it is ordinarily encountered, for example those of liver parenchyma, is sufficiently shown by its appearance in connective tissue exposed to carbon dioxide as just described.

The color of the organs of animals vitally stained with brom cresol purple places them in two groups,—those which are definitely alkaline to the indicator, as shown by a purple or blue coloration, and those which appear more or less acid, being stained yellow or green. Before a scrutiny of this grouping is undertaken the outcome of staining with other indicators will be described.

Chlor Phenol Red.

Chlor phenol red[4] has nearly the range of brom cresol purple, but is free from dichromatism, and, in the amount required for vital staining, appears non-toxic for mice. The material I have employed is rose-purple at pH 6.24, yellow-pink at 5.75 and 5.91, orange at 5.59, and yellow at pH 5.29, when distributed in phosphate buffer mixtures at dilutions yielding approximately the color intensities ordinarily encountered within the tissues. When ten or twenty times as much indicator is used the color distinctions are far less satisfactory. The solutions are now purply red above pH 5.91, and orange-red to ruddy orange down to pH 4.6 at which they are a deep orange-red. The blood of stained animals, wherein alone the indicator is ordinarily greatly concentrated, never appears sharply purple under the microscope, as in the case of brom cresol purple, but only red, whence it follows that histological distinctions are less clear-cut than with this phthalein.

Mice of 18 to 30 gm. receiving an intraperitoneal injection of 0.6 gm. of a 2 per cent solution of chlor phenol red become colored old rose within a few minutes and remain so for the better part of 2 hours, after which the coloration rapidly fades and by the next day is gone. The mouse acts normally when the staining is at its height. The urine voided is a deep purply red.

[4] Cohen, B., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 199.

The ear of the unanesthetized animal, as viewed between glass slides, is pronouncedly old rose, even when the blood is pressed out.

The white cells and platelets of the *blood* are not stained, but the erythrocytes appear abnormally red, and, on the application of NaOH to a thick preparation of them, become rose-purple, far more so than does the serum, thus showing the presence of indicator.

The cartilage, bones, and tendons are all rose-purple, the distribution of the coloration and its relative intensity being practically the same as in the case of brom cresol purple.

The lymph nodes appear purply *in situ*, but when they are incised and the lymph expressed the major portion of the coloration is seen to be due thereto. Tests with NaOH show that the pale yellow lymphoid tissue contains some of the indicator, for it then becomes rosy.

The connective tissue everywhere is a notable rose-purple. Under the microscope the hue is less vivid than the bluer purple of brom cresol purple; and the color distinction between the connective tissue and parenchyma of some of the organs is in consequence less marked. The nerve sheaths are old rose.

The nerves, the epidermis, the splenic tissue (except the connective tissue scaffolding) and the *brain* all fail to stain.

There is so little of the phthalein in the *adipose tissue* that this usually has the normal hue, but when a great deal of the indicator has been brought directly into contact with it, as happens within the abdomen, it shows regions stained yellow, in contrast with the ruddy purple of the indicator solution originally injected.

The voluntary muscle fibers appear slightly yellower than normal, and sodium hydroxide changes them to rose as it penetrates through the cut ends. The blood and lymph should be squeezed out of the tissue bit prior to this test by rapid pressure between folds of neutral gauze.

The parenchymal cells of the *pancreas* appear brilliant yellow, turning rose-purple with alkali.

The kidney cortex sometimes appears yellow under a low power through a tinting of the majority of the tubules; but, even so, pale purple ones can be discerned here and there. The medulla, on the contrary, is an outspoken purple. In some instances the majority of the cortical tubules are purply.

The gall bladder bile is, in general, a dark purply red, though that which lies between folds of the mucosa is sometimes an intense orange with the indicator. The wall of the viscus is usually stained purple, but in one instance it was brightly yellow, and became purple only through a secondary change as it lay steeped in alkaline bile after its removal.

The liver parenchyma is stained a definite if pale yellow. Rosy bile fills the larger ducts.

The suprarenal bodies contain not a little of the dye in the yellow, acid, form, as is demonstrated by the application of weak NaOH. Most of the phthalein lies in the cortex, though the medulla, owing to its translucency, appears the yellower.

The heart muscle contains almost none of the dye and that little in the yellow form.

The lung tissue is a pale rose.

Chlor phenol red does not stain quite so many of the tissues as brom cresol purple; but the colors it assumes in those which it does stain are such as one would expect were the findings with brom cresol purple an expression of actual conditions within the tissues. The same two groups of tissues, colored as if distinctly acid and relatively alkaline respectively, may be discriminated by its means as by brom cresol purple.

The application of sodium hydroxide to tissues stained with phthaleins, to test whether these are present in the acid form, is a method involving a pitfall.

Alkalinization may effect not alone a change in color, but so great an intensification in one previously present that it may, for the first time, become noticeable. This is especially true in the cases of brom cresol purple and chlor phenol red. The secondary purpling of tissues derived from vitally stained animals, but themselves only dubiously stained, tells little as regards the initial color of the phthalein within them. Only when tissues obviously yellow turn purple with hydroxide is a conclusion warranted that the indicator was originally present in the acid form.

Brom Cresol Green, Brom Phenol Blue, and Thymol Blue.

Methyl red, belonging to another group of indicators but occupying an important place in Clark's series, fails to produce an intense vital staining. In its stead a phthalein, brom cresol green, recently studied by Cohen,[4] has been employed.

This dye is well tolerated by mice. Its range, under controlled conditions, is variously given as from pH 4.0 to 5.6 or 6.0. The material I have used is a definite blue at pH 5.0 in Northrop's buffer or Walpole's acetate buffer, becoming green as acidity increases, until at pH 4.0 it is a frank yellow.[5] Animals injected with solutions

[5] When mentioning the range of brom cresol green in a previous paper[1] it was erroneously stated that phosphate buffer mixtures were employed to test it. The range of these does not extend sufficiently far to the acid side for such a purpose. Northrop's buffer was used.

of the sodium salt titrated to approximate neutrality become brilliantly blue on ears and tail, and void an intensely blue-green urine; but not a few of the tissues fail to stain, especially the adipose tissue, voluntary muscle, heart muscle, bone, lymph glands, lungs, and pancreas. The epidermis, cartilage, connective tissue, kidney, liver, and tendons are all rendered more or less pronouncedly blue. Much of the dye is excreted through the bile, which is deep blue with it.

The range of the indicator evidently lies too far to the acid side for usefulness in the determination of tissue reactions, save in special instances, as that of the macrophage granules.[2] The same holds true of brom phenol blue which is yellow at pH 3.2 and blue at pH 4.2 under controlled conditions.[4] Mice receiving the sodium salt are rendered deep blue, secrete a urine of even deeper shade, and within a few hours recover the normal hue again. Nowhere in the body, save in some of the macrophage granules on occasion, and in the stomach contents, is the coloration other than blue. At both situations it is pale yellow, changing to blue with alkali.

The observations with thymol blue have already been reported.[1] The tissues are not alkaline enough to come within the alkaline range of this phthalein, nor acid enough to be within the acid one. There is a pronounced yellow staining, such as is indicative of a pH of less than 8.4 and more than 2.6, under controlled conditions.

The Indicators in Bile, Urine, and Intestinal Contents.

All of the indicators escape in quantity into the bile; and for some that are most diffusible, judging from their behavior in the organism, this is the main avenue of elimination. That they retain the characteristic color range is evident from the hue of the secretion, and from special tests in the case of brom cresol purple.[1] Liver bile containing thymol blue is yellow; it is red in the cases of cresol red and phenol red, purple with brom cresol purple, rose-purple with chlor phenol red, and blue with brom cresol green and brom phenol blue. To judge from all this, the liver bile of the mouse is definitely, if slightly, alkaline, like that of other laboratory animals. The bladder bile is, on the contrary, frequently somewhat acid, especially that next to the mucosa,—witness the findings with brom

cresol purple (*q. v.*),—as would follow from what is known of the modifying influence of the viscus.[6] That the wall of the gall bladder also seems to have an acid reaction is surprising, not only because it so largely consists of connective tissue but because the change it effects in the reaction of stored bile supposedly comes about through the removal of constituents that are predominantly alkaline in character.

The contents of the pyloric portion of the stomach are of a brilliant yellow hue with the phthaleins, as would follow from the prevailing acidity. Whether the dyes are directly secreted into the organ or reach it from the duodenum has not been determined. The small intestines usually contain much semifluid material heavily stained with indicator from the bile, and the feces are colored as well. That some slight secretion takes place directly into the large intestine is attested in the case of brom cresol purple by the recovery therefrom of a pale purple mucus soon after injection of the indicator, before the colored chyme has progressed as far as the cecum. Within an hour, ordinarily, after the intraperitoneal injection of almost any one of the phthaleins, the intestinal contents are stained this far down; and indicators suited to the conditions, as for example, phenol red and brom cresol purple stain the chyme differently at different levels of the gut, in accordance with the local differences in reaction. When brom cresol purple has been given by mouth it appears in the bile, evidencing absorption.

In animals vitally stained with phenol red, chlor phenol red, or brom cresol purple, striking individual differences in the color of the renal cortex are to be observed which are doubtless referable to differing states of functional activity.

Frequently the color of the medulla contrasts with that of the cortex as a whole, being, for example, purple with brom cresol purple, when this is yellow. Stieglitz[7] has noted that the reaction of the renal cortex, as indicated by the color it assumes in dogs injected with azolitmin, sodium alizarinate, or neutral red, tends ordinarily to be the opposite of that of the urine, alkaline when this is acid and *vice versa*. In general, the principle would seem to hold for phthalein-stained mice. But it

[6] Drury, D. R., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1924, xxxix, 403.
[7] Stieglitz, E. J., *Arch. Int. Med.*, 1924, xxxiii, 483.

is obviously defective in that it involves a disregard of local variations in reaction within the cortex. Differences in the color of neighboring cortical tubules when stained with phthalein are frequent, recalling those met in litmus-stained animals.[8]

Evidences of Pathological Alteration in the Tissue Reaction.

Preexisting lesions are often brilliantly picked out in color when a phthalein has been given.

They are especially well seen in mice receiving brom cresol purple. Individual fibers of striped muscle affected with sarcosporidiosis are then purple in contrast with normal ones. New formed granulation tissue may be pale purple, save where it borders on necrotic foci and here a chalcedony yellow. The red cells in vessels compromised in such foci, may have this latter hue instead of the usual purplish orange. Old scars in the renal cortex may be purple against a yellow background. In an instance of hepatic disease the giant cells, scattered here and there, were a dark amber, turning to livid purple with alkali, whereas the reactive tissue associated with them was green, not purple like the more normal connective tissue nearby. Casts situated within a kidney cortex of yellow-green hue may themselves, be purple, or a deep brownish yellow.

The administration of a highly concentrated solution of brom cresol purple, or one that has been autoclaved, may kill some cells, especially such as come in contact with it in the peritoneal cavity, as also some within the organs principally excreting the dye, namely the liver and kidney. The state of affairs is obvious from the altered character of the staining. The cell nuclei are now deeply colored by the indicator, as does not happen with living elements, and the hue of the cytoplasm, while lighter, is also relatively intense.

Supravital Changes in Reaction.

Significant alterations are to be observed in the hue of some of the tissues within a few minutes after death of the animal.

A relative acidosis develops post mortem which affects the color of the hairless surfaces, not only by changing the hue of the blood but that of the connective tissue as well,—facts readily discernible with phenol red and brom phenol red.[1] Even in animals stained with brom cresol purple there occurs a change in the color of the connective tissue from purple to blue-green. Certain other tissues, among them the heart muscle, adipose tissue, and parenchyma of the pancreas, become pronouncedly more yellow. The conditions leading to such a change in the undisturbed organs are, of course, highly complex.

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[8] Rous, P., *J. Exp. Med.*, 1925, xli, 379.

For the detailed study of supravital alterations it is best to remove small portions of the organs under oil, and to place them while still surrounded by it between mica slips. Systematic observations of the sort, at room temperature, have disclosed certain characteristic phenomena.

In general it may be said that the group of organs having a relatively acid reaction to begin with, as indicated by the vital staining, all seem rapidly to become more acid, whereas those appearing less acid or slightly alkaline undergo no change for a long time. Thus, on the one hand, bits of the liver and pancreas, of muscle, adipose tissue, and kidney cortex, which, during life, are greenish yellow with brom cresol purple, all turn a saffron yellow within a few minutes after removal. The fact that the change in color largely comes about through an alteration from purple to yellow of the associated, deeply stained, connective tissue, blood plasma, and lymph does not affect the significance of the phenomenon. No such change occurs, even after an hour, within bone, connective tissue, tendons, cartilage, and epidermis, members of the relatively alkaline group, and the alteration is but slow when they contain phenol red, which is suited by its range to disclose the slightest shiftings in reaction. The difference between the groups is evinced in yet another way. On exposure to air those tissues which appear relatively acid during life do not, in general, undergo any prompt color alterations indicative of pronounced changes in the direction of alkalinity but maintain nearly the initial hue, whereas the ones with an initial reaction closer to neutrality rapidly change color, becoming alkaline to all appearance. In this connection the influence of ventilation from exposed connective tissue surfaces upon the local reaction[1] may be recalled.

GENERAL DISCUSSION.

For the study of natural phenomena one may either develop a method to suit the conditions, or force the latter to suit a method. The literature on the reaction within living tissues illustrates in an enlightening way the difficulties inherent in both procedures.

The work with exact physical-chemical methods need not be reviewed in detail. It has been briefly analyzed in a previous paper,[1] as in those of Gräff,[9] and of Schade and his associates.[10] Vlès has recently[11] summarized a portion of the published data, together with the results of some scattered attempts to determine the

[9] Gräff, S., *Beitr. path. Anat. u. allg. Path.*, 1923–24, lxxii, 603.

[10] Schade, H., Neukirch, P., and Halpert, A., *Z. ges. exp. Med.*, 1921, xxiv, 11.

[11] Vlès, F., *Arch. phys. biol.*, 1924, iv, 1.

hydrogen ion concentration within cells by vital coloration. He himself has tested, by several physical-chemical methods, the reaction within frozen and ground mouse tissues.[12] He states that in the substance of voluntary muscle fibers there is a hydrogen ion concentration averaging about pH 6.0. Many events, as for example, infection, the growth of tumor grafts, and the genital evolution in the female, are stated to affect the reaction. Such findings are in many ways unique. The general experience has led a recent reviewer[13] of neutrality regulations within the body to conclude that the organism must dispose of acids "sufficiently rapidly so that the hydrogen ion concentration of the blood and tissues does not rise above $1 \times 10^{-6.9}$."

The older work on the staining of mammalian tissues with indicators has been considered in a previous paper.[8] During the last 2 years there have been several attempts to utilize the phthaleins for this purpose. Gräff[9] has removed bits of organs from dead animals and human beings into drops of the indicators placed against a white surface, and has drawn inferences as concerns the tissue reactions from the ensuing color changes. He gives a table according to which the reaction of all of the organs is well above pH 7.0—a finding with which one need not quarrel when the conditions are considered. In the laboratory of Vlès, Reiss[14] has placed mammalian tissues in solutions of indicator and noted the colors they assume when crushed. Very recently, too, he has injected living mice with quantities of brom cresol purple and brom thymol blue insufficient for a general staining, and has found that they accumulate in the liver in recognizable amount, coloring the bile and the intestinal contents.[15] His findings in crushed tissues stained *in vitro*, together with Vlès' findings by physical-chemical methods lead him to believe that the larger organs have an acid reaction during life. In this connection one may recall that Michaelis and Kramsztyk[16] demonstrated, in 1913, with the aid of the potentiometer, that acidity develops rapidly within some of the larger viscera after death. And the least trauma to voluntary muscle causes acid to form.[17]

The fact has been known for some time that tissues will grow *in vitro* in a medium acid to litmus;[18] and the fact is now well authenticated through indicator tests that they survive surprisingly wide variations in pH.[19]

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[12] Vlès, F., and de Coulon, A., *Compt. rend. Acad.*, 1924, clxxix, 82; *Arch. phys. biol.*, 1924, iv, 43.

[13] Wilson, D. W., *Physiol. Rev.*, 1923, iii, 295.

[14] Reiss, P., *Arch. phys. biol.*, 1924, iv, 35.

[15] Reiss, P., *Compt. rend. Soc. biol.*, 1924, xci, 1433.

[16] Michaelis, L., and Kramsztyk, A., *Biochem. Z.*, 1914, lxii, 180.

[17] Fletcher, W. M., and Hopkins, F. G., *J. Physiol.*, 1906–07, xxxv, 247.

[18] Rous, P., *J. Exp. Med.*, 1913, xviii, 183.

[19] Lewis, W. H., and Lewis, M. R., A text-book of general cytology, Chicago, 1924, 385.

Stieglitz' observations on the acid state of the renal cortex, when secreting alkaline urine, have already been referred to. The indicators he employed have ranges that barely extend into acidity. This is true, as well, of litmus which effects a widespread, red, vital coloration, owing to storage of it within cell granules. Not a few data on reaction conditions within the organism have been obtained with its aid, however.[2] The granules of macrophages, in which it is laid down in the red form, will, on occasion, store phthaleins also; and they then show colors which, under controlled conditions, would indicate an acidity at least as great as pH 3.0. Marshall and Vickers have noted that the convoluted tubules of animals injected with phenol red are colored yellow;[20] and Sharlit and Scheer[21] and Memmesheimer[22] have painted the cleansed skin of human beings with alcoholic solutions of the phthaleins and found colors indicative of a pH between 5.2 and 5.8.

To sum up, it can be said that the advocates of exact methods in the determination of the reaction within living organs have learned much that is of advantage about sources of error and impediments to observation. The group of workers who have chosen their method to suit the conditions,—the indicator method, by process of exclusion, —have made observations with ease but without assurance of certainty. No systematic examination of conditions throughout the body has been carried out heretofore.

The observations of the present paper were made under circumstances which excluded common sources of error, for example, exposure to air, asphyxia, postmortem change, etc. The findings in tissues swiftly removed from the living animal have been controlled by direct inspection of them *in situ*, save in the case of organs so vascular that the blood has obscured the color. The significance of what has been found will now be discussed.

Nature of the Phthalein Staining.

That the phthaleins pass into the lymph is evident, not only from their great diffusibility and from the experiments in which animals were washed free of blood,[1] but from the brilliant tint of the fluid pressed out of lymph nodes. That they penetrate some, at least, of the cells is plain from the abundant excretion of them into

[20] Marshall, E. K., Jr., and Vickers, J. L., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 2.

[21] Sharlit, H., and Scheer, M., *Arch. Dermatol. and Syphilol.*, 1923, vii, 592.

[22] Memmesheimer, A., *Klin. Woch.*, 1924, iii, 2102.

bile and urine. The cells of the parenchyma of the liver and of the renal cortex appear diffusely colored. So, too, does many another parenchyma, as for instance that of the pancreas. There is usually no difficulty in telling whether cells have stained, so outspoken is their hue; but sometimes one cannot be sure that the phthalein has penetrated into the interior of the cell and not merely conferred color through a fixation upon its surface. For this reason, among many, it is best to speak of the reaction within tissues rather than within cells. Certain cells there are which appear closed to the phthaleins, or which take them up in such slight amount that no staining can be made out. The lymphoid cells and those of bone and of cartilage are among these, as are the leucocytes of the blood and, not impossibly, fibroblasts. Intercellular substances, especially those of connective tissue, of tendons, of the cartilage of most regions but not all, and well vascularized bony matrix, as distinct from that which is dense and lamellar, become diffusely stained by all the phthaleins, as by litmus, a colloidal indicator which colors cells of a few sorts only, and these in consequence of a segregation into granules. It is as if there were little power of election for the intercellular materials as regards the staining, whereas one exists in the case of the cells.

Certain organs take up so little of the indicators that the hues proper to the latter prevail but slightly over the ordinary cell color. This is true of voluntary muscle more especially. Yet that penetration into the fibers has occurred can be demonstrated by applying alkali to them, when they become intensely colored throughout, the phthalein changing from a yellow obscured by the natural hue of the muscle to purple in the case of brom cresol purple, and rose color in that of chlor phenol red. It might be thought that the staining is secondary, coming about by a spread from the blood or lymph of the dye, now intensified in color; but the even distribution of it within the fiber and its development even when the free fluid has been pressed out of the muscle bit, preclude such a possibility.

Errors of Interpretation.

The color assumed by a stained tissue is the resultant of a complex of variables, however much of the indicator is present.

The hues assumed by the blood and lymph and connective tissue are frequently wholly different from that of the associated parenchyma, notably in the cases of brom cresol purple and chlor phenol red. It follows that discrimination with the

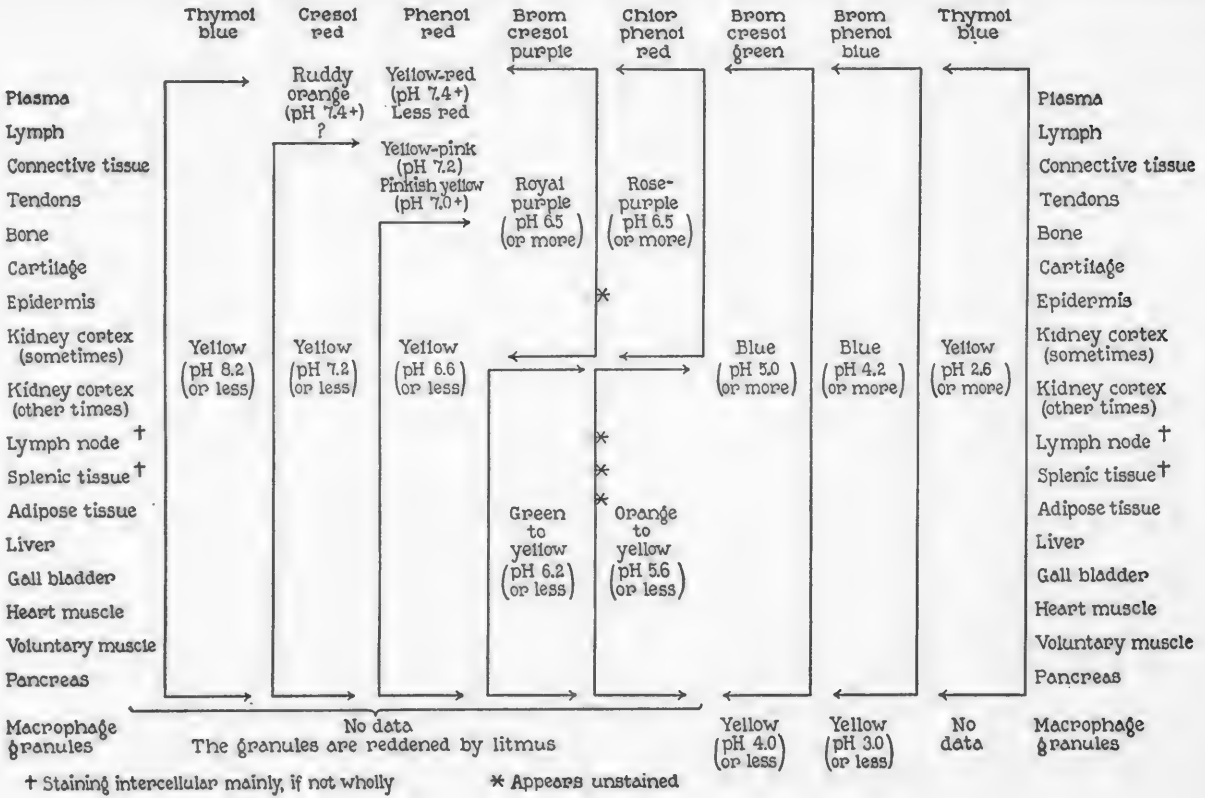
microscope is often necessary. The color of thin layers of tissue frequently differs from that of thick ones for the same reason that a buffered solution of indicator in a narrow test-tube looks different from that in one of wide bore. The additional complication of dichromatism is present in the case of brom cresol purple.

Kolthoff[23] states that the phthaleins yield unreliable results in the presence of proteins. Protein and salt errors are to be expected of any substances with indicator properties; and the best one can do, as Clark has said, is to use those least affected. The phthalein indicators are, from this point of view, the most satisfactory available. In a subsequent paper their defects will be inquired into, and an attempt made to interpret the vital coloration with them. Meantime no harm can come from a scrutiny of the tissue staining in its immediate aspects. The data recorded in my previous papers will be lumped with those of the present communication. One may disregard, for the time being, the potential complications involved in a toxicity of the indicators, in the influence of the anesthetic, in trauma to the tissues during removal, and in supravital changes. The findings with the phthaleins as a group are set forth in Chart 1 according to the order in which they stand in series, from thymol blue in its alkaline phase at the left, to the same indicator in its acid phase at the right.

Relative Reactions within the Organs.

None of the tissues, or body fluids, is sufficiently alkaline or acid to come within either of the two ranges of thymol blue; the reaction of all those stained,—and they are many,—would appear to lie somewhere between pH 8.2 and 2.8. With cresol red the blood has a different color from the tissues, one that under controlled conditions would be indicative of pH 7.4 approximately. The staining of the latter generally shows that the reaction within them lies beyond the range of cresol red on the acid side, that is to say at or below pH 7.2, assuming that the face value of the findings is the real one. With phenol red the blood again has a color indicative of pH 7.4, and now two tissues have hues within the phthalein range, namely the connective tissue and tendons. Apparently the hydrogen ion concentration within both lies between pH 7.0 and 7.2, that is to say on the acid side of the range of cresol red, as is the actual case. The tests tell nothing of the other tissues save that the reaction within them lies beyond the range of phenol red on the acid side.

[23] Kolthoff, I. M., Der Gebrauch von Farbenindicatoren, Berlin, 1923, 49.



† Staining intercellular mainly, if not wholly

CHART 1.

With brom cresol purple the reaction of the organs, as expressed in the color, enables one to place them in two groups, those which have, apparently, an interior pH of 6.5 or more, as attested by hues on the alkaline side of the indicator range, and those seemingly more acid. In the first group one finds the connective tissue and tendons, as one would expect, and bone, cartilage, and epidermis as well. It is interesting to recall that these are tissues which stain diffusely blue with litmus. The hue of the kidney cortex stained with brom cresol purple sometimes places it within the relatively alkaline group, and again in the acid one. Always one finds in this latter the liver, gall bladder, pancreas, heart muscle, and voluntary muscle, the adipose tissue, and, though the staining is but light and perhaps wholly intercellular, the lymph nodes and the Malpighian corpuscles of the spleen. The hydrogen ion concentration in all these would seem to be as great as pH 6.2, and often greater. The parenchymal cells of the pancreas, in special, appear notably acid. With chlor phenol red many of the findings can be confirmed, though not all, for it stains less widely than brom cresol purple, and there is a zone near the middle of its range where the hues, especially as viewed within tissues, are indeterminate. The connective tissue, tendons, bone, and cartilage, when stained with this indicator, have an apparent reaction above pH 6.5; whereas the liver, gall bladder, kidney cortex (sometimes), heart muscle, voluntary muscle, and pancreas would seem to be at least as acid as pH 5.6. The epidermis, adipose tissue, lymph nodes, and splenic tissue proper, as distinct from the connective tissue scaffolding, all fail to stain. The reactions of the blood and lymph lie beyond the ranges of chlor phenol red and brom cresol purple on the alkaline side, as their hue shows.

The indicators of still more acid ranges color the tissues in such wise as to indicate that the reaction lies beyond the ranges of all on the alkaline side, that is to say at or above pH 5.0 in the case of brom cresol green, at or above pH 4.2 in that of brom phenol blue, and at or above pH 2.6 with thymol blue. An exception is to be noted for the macrophage granules. These, which appear acid to litmus, are likewise acid to brom cresol green, and even in some instances to brom phenol blue,[2] indicating a pH as low as 3.0 if not lower. The stomach contents likewise has the color associated with acidity.

In this ranging of the tissues according to the phthalein staining the pH scale has proved indispensable. But the use made of it must not be taken as implying a belief that the actual reaction prevailing within the tissues is the same as the apparent one. The discrepancies between the two have yet to be studied. Good reasons exist, nevertheless, for the supposition that the separation of the tissues into two major groups of notably different hydrogen ion concentration is based on reality.

The less acid group of tissues, some among which appear to be definitely if very faintly alkaline, have all a low metabolic activity. According to Warburg,[24] the metabolism of connective tissue is minimal; and it is this tissue which appears to be the most alkaline of all. Warburg was not able to measure either respiration or glycolysis in it with certainty. All of the group, excepting the epidermis, are made up of a great deal of intercellular substance with relatively few cells. It is principally the intercellular substance which stains; and this is diffusely penetrated by even so colloidal a material as litmus. One may suppose the alkaline body fluids to penetrate it with at least equal ease. Most of the more acid group of tissues by contrast are notably cellular, and all have a high metabolic activity, except perhaps the adipose tissue and the gall bladder which two are endowed with special functions that may account for the acidity observed. When a piece of liver or pancreas or muscle, stained with brom cresol purple or chlor phenol red, is extirpated and placed in oil under a mica cover-slip, acid is rapidly formed within it, as shown by changes in color of the associated connective tissue and blood. No such alteration occurs in bone, cartilage, tendon, or connective tissue, even after a long time; and it takes place but tardily in the two tissues last mentioned when they have been colored with phenol red. When stained with this indicator and exposed to air, all of the group just mentioned, except the epidermis, which keeps its hue, become rapidly more alkaline, as the color changes show, whereas the tissues of the relatively acid group are affected slightly when at all and only very slowly when brom cresol purple has been used. The staining of the acid group is essentially one of the cells. What is known of these elements does not encourage a belief that the alkaline body fluids go in and out of them as in and out of the intercellular fabrics.[25]

From the foregoing one obtains the impression that the highly cellular tissues make their own conditions, so to speak, one of these conditions being a relative, and probably an absolute acidity; whereas those with few cells in a large bulk of matrix are vulnerable to external influences, control their own conditions only in so far as the activity of the scattered cells extends, and in consequence of one or both of these circumstances have a reaction only slightly acid at most, when so at all.

If the color differences with the phthaleins turn out to be the expression of actual differences in the reaction within the organs, much that is now obscure in physiology and pathology can be explained,—or explained away. As illustrating the point, one need

[24] Warburg, O., *Naturwissenschaften*, 1924, xii, 1131.
[25] Jacobs, M. H., General cytology, Chicago, 1924.

only think of the problem offered by the normal inactivity of certain enzymes which living cells possess, and that of the localization of certain species of pathogenic bacteria in special organs. But it is not too early to remark, in such connection, that the reaction prevailing within a given tissue, however much it may differ from that elsewhere, must be thought of as, after all, but one among the host of varying attributes whereby tissues are distinguished from one another.

Further studies of the distribution throughout the body of the phthaleins will assuredly bring out facts affecting the general theory of vital staining. These substances color some tissues, for example the red cells of the blood and the pancreatic parenchyma, which are unaffected by the acid dyes previously studied.[26] Diffuse staining, such as the phthaleins accomplish to a preeminent degree, has thus far had little attention from investigators and the laws regulating it are largely unknown.[27]

Supplementary Note.

The findings with brom phenol red described in a previous paper of this series[1] have designedly been omitted from present consideration. The material employed under the name of brom phenol red to obtain those findings had a pH range wide of the one given by Cohen,[4] a fact stated in reporting upon them. The discrepancy, and the results of a recent comparison with other samples furnished as the phthalein, which has been carried out in this laboratory by Dr. D. R. Drury, have led us to question the purity of the product first employed. The samples now procurable as brom phenol red have a pH range practically identical with that of chlor phenol red.

SUMMARY.

In continuation of the observations on vital staining by means of phthalein indicators, reported in a previous paper, some members of

[26] von Möllendorff, W., Farbenanalytische Untersuchung der Zelle, in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 2nd edition, 1924, ii, 273.

[27] von Möllendorff, W., *Ergebn. Physiol.*, 1920, xviii, 141.

the group with acid ranges have been employed. The results accord closely with those already obtained, in pointing to the existence of characteristic differences in the reaction within the organs. Roughly speaking, these latter fall into two groups. Within the members of the one a noteworthy acidity would appear to prevail, whereas in those of the other acidity seems slight at most, and in a few instances signs of a faint alkalinity are to be met. In general the "acid" organs are such as have a great metabolic activity whereas the "neutral" group manifest but an inconsiderable one. All of the tissues examined seem to be relatively acid compared with the blood plasma.

The findings with the phthaleins as a group are discussed.

SKIN REACTIONS TO SIMULTANEOUS TREATMENTS WITH RADIANT HEAT AND SOFT X-RAYS.

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In the course of experiments in which guinea pigs were exposed to x-rays it was observed in several instances by one of us (Hawkins) that whereas a given exposure to the rays, filtered through very thin cardboard, produced no visible effect on the skin even after several days, the same exposure to unfiltered rays in otherwise similar circumstances was followed a few hours later by the appearance of a burn. The difference in intensity of the x-radiations, filtered and unfiltered, amounting to 3 or 4 per cent of the ionizing power in air, can scarcely account for such widely different effects. The nature of the burns and the promptness with which they appeared suggested that they might have been caused in part at least by radiant heat from the tube. That healing did not occur promptly and properly indicated, however, that there had been some aggravation by the x-rays. In order to determine whether or not the combined action of x-rays and radiant heat has a more profound effect than either agent alone we have made some experiments on guinea pigs in circumstances such that the two kinds of radiation could be administered separately or together.

Method.

The x-rays were supplied by an outfit previously described,[1] the output of which is known to be constant in quality and intensity. The tube and the animal occupied two separate compartments of a lead-lined cabinet, the rays passing through an aperture covered with a filter of thin bristol board. The compartment containing the tube was sufficiently ventilated by means of a fan to keep the filter and other parts of the partition cool, thus preventing the heat of the

[1] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

tube from reaching the animal. The tube was operated in all cases at 30 kilovolts and 22 milliamperes.

Radiant heat was supplied in some cases by electrically heated coils of nichrome wire and in others by an electric soldering copper. The source of heat was placed about 3 cm. above the abdomen of the animal, close to, but not actually in, the path of the x-rays. The position relative to the skin area to be exposed, and to the cabinet, was carefully maintained throughout each series of experiments. No attempt was made to measure the rise in temperature of the skin; the conditions of heating were carefully reproduced, however. The copper, when used, was preheated for the same length of time in every case, and both copper and coils were operated at constant voltage. All exposures were begun with the cabinet at room temperature and the cabinet doors were kept closed during the experiments to insure constant air current conditions. The fact that each exposure of heat, in the following experiments, lasted 1 minute longer than the corresponding x-ray exposure was necessary on account of the technique of handling the x-ray apparatus. Before each experiment the intensity of the heat was adjusted on the basis of a few preliminary trials to the critical value for producing little or no burn.

The technique of holding the animals in place and at a definite target distance has been described.[1] The abdomen was shaved before treatment and covered with lead-filled rubber except for the area, 7 by 5 cm., to be exposed. For further protection from the heat and Roentgen radiations a lead sheet with a hole corresponding to the one in the lead rubber was placed over the whole animal.

EXPERIMENTS.

Experiment 1.—An area on the abdomen of each of six guinea pigs was exposed for 11 minutes to the heat from a nichrome coil held about 3 cm. above the surface of the skin. A second series of seven pigs received a combined exposure to x-ray and heat, the duration of the exposures being 10 and 11 minutes, respectively. A third group of seven animals received a 10 minute exposure to x-ray alone. The target distance for the x-ray exposure in both of these groups was 35.4 cm., which gives, with the other factors, about 90 per cent of the estimated critical dose for producing slight scaling of the skin. For the control exposures of heat and x-ray alone the two agents were administered separately under conditions identical with those obtaining when given together.

Among the animals exposed to heat alone two of the six pigs developed burns which appeared immediately after the treatment but were healed completely between the 10th and 14th days. Of the seven animals receiving the combined x-ray and heat six developed extensive burns which did not heal completely till between the 42nd and 59th days after treatment. None of the animals exposed to x-ray alone showed visible skin changes (Chart 1).

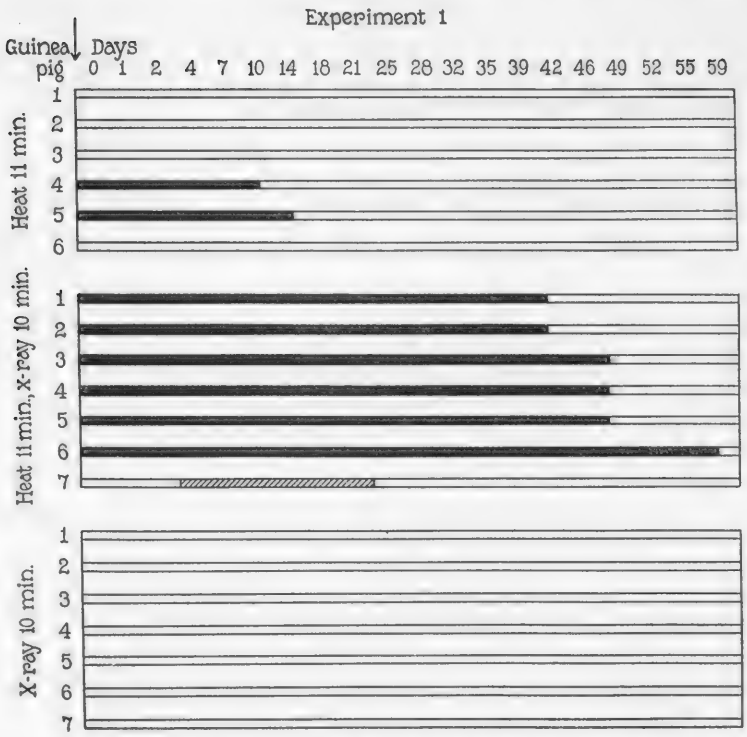

CHART 1. Each line represents the history of a single individual animal from the time of exposure till the end of the experiment. The heavy lines indicate burns; the cross-hatch, scaling but otherwise intact skin; and the unshaded line indicates no reaction, or a healed lesion in the skin.

Experiment 2.—An area on the abdomen of each of ten guinea pigs was exposed for 11 minutes to the heat from an electric soldering copper held 3 cm. from the skin. Another lot of eleven animals were exposed in a similar fashion to heat for 11 minutes and x-ray for 10 minutes, the exposures being made simultaneously. As the x-ray dosage used for this experiment was the same as that of Experiment 1, no control was included for the effect of this agent alone.

All of the heated animals with two exceptions developed burns but these did not appear till 24 hours after exposure and were completely healed between the 10th and 23rd days. Among the eleven animals receiving the combined treatment all developed burns, which, with two exceptions, appeared immediately

after exposure. The healing in this group was somewhat irregular. In three animals it was complete between the 21st and 28th days, but in the remaining eight not until between the 42nd and 59th days (Chart 2).

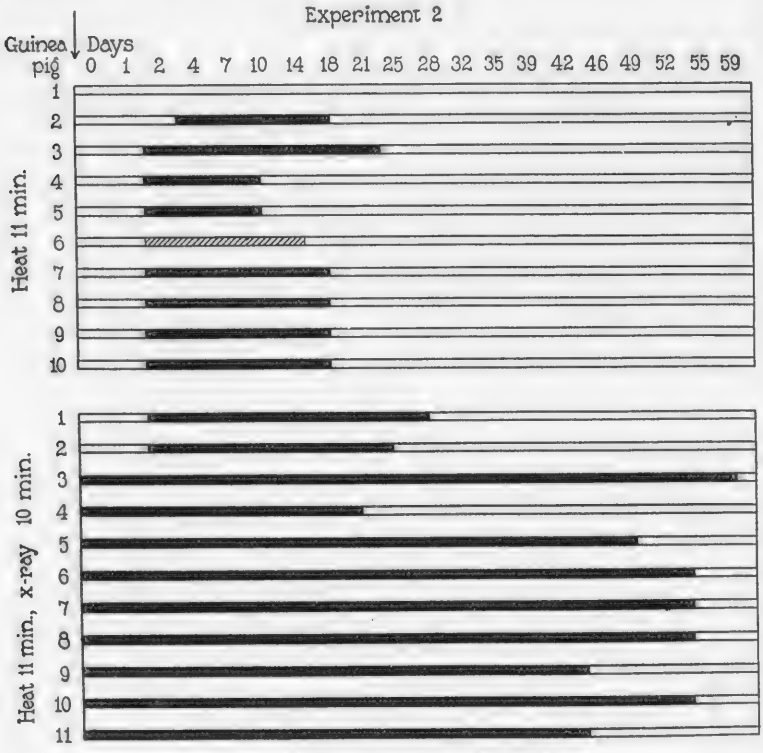

CHART 2. See explanation of Chart 1.

Experiment 3.—The source of heat in this experiment was the same soldering copper as that used in Experiment 2, but the distance from the skin was increased to 4.5 cm. and the length of exposure to 16 minutes. Of the five animals exposed to this dosage of heat only one developed a burn and this appeared the 2nd day and was healed by the 14th day after treatment. The other four guinea pigs all showed slight scaling but no break in the skin.

There was a slight change also in the x-ray dosage in this experiment, the target distance being increased to 36.7 cm. and the time to 15 minutes. This dose was sufficient to cause some scaling. The combined treatment with the two agents produced burns in all of the five animals exposed. The lesions appeared the 1st

day but the healing showed some irregularity, the process being complete in one animal by the 21st day, in two others by the 28th and 32nd day, respectively, and in two only at the 59th day (Chart 3).

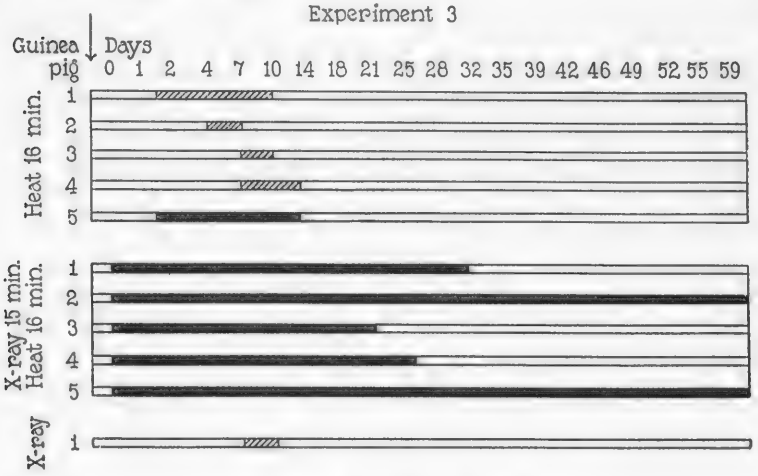

CHART 3. See explanation of Chart 1.

From these results it is seen that there is a marked difference between the developments in animals receiving either heat or x-radiation alone, and those receiving both radiations simultaneously. The x-rayed animals used as controls in Experiments 1 and 2 showed no skin reaction throughout the observed period, the hair growing in on the exposed area by the 14th day after treatment. In Experiment 3 the x-ray dosage given was sufficient to produce slight scaling with no erythema. The animals which were exposed to heat radiation alone, exhibited slight burns in 50 per cent of the animals treated. The burn appeared as a small white thickened area on the 2nd or 3rd day after treatment, which broke down and ulcerated on the 4th or 5th day and healed completely between the 10th and 14 days after exposure, leaving practically no scar. The irregular results from the heat treatment were to be expected because many factors were involved and the estimated dose was close to that which would regularly produce a burn.

In the experiments in which the guinea pigs were exposed to heat and x-radiations, simultaneously, all the animals developed well marked burns except one which had persistent heavy scaling for 2 weeks. The skin of these animals showed small hemorrhagic spots in the exposed area immediately after treatment. 1 or 2 hours later a large edematous area had appeared which broke down in 2 or 3 days. The lesions resulting were similar to those produced by heat radiation alone but were much more extensive. They healed much more slowly, the majority taking from 35 to 50 days, and when healed left a thick scar.

DISCUSSION.

No interpretation of the phenomenon is ventured. Whether one sort of radiation sensitizes the tissue to the action of the other can be learned only by giving the treatments in sequence. Experiments of this kind are now in process.

It is of interest to note that Rohdenburg and Prime[2] working with certain transplantable mouse and rat tumors have found that the combined effects of a certain exposure to heat and x-rays *in vitro* were lethal, although either exposure alone was without effect. A phenomenon somewhat similar perhaps has been observed by Bovie and Daland,[3] who have shown that by an exposure to fluorite rays *Paramecium caudatum* is rendered extremely sensitive to heat, so that it is injured or killed by an amount of heat which would not affect a normal unradiated individual.

SUMMARY.

Guinea pigs have been exposed to suberythema doses of soft x-rays, to radiant heat of intensity about critical for producing slight burns, and to both radiations simultaneously. No erythema was produced in the skin of the animals exposed to x-rays alone and only slight burns resulted in 50 per cent of the animals exposed to heat radiation alone. The animals exposed to heat and x-radiation simultaneously developed well marked burns which healed much more slowly than those produced by heat alone.

[2] Rohdenburg, G. L., and Prime, F., *J. Cancer Research*, 1921, vi, 101.
[3] Bovie, W. T., and Daland, G. A., *Am. J. Physiol.*, 1923, lxvi, 55.

THE LIVER AS A SOURCE OF BACTERIAL AGGLUTININ.

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The locus of antibody formation has not been definitely determined. The opinion at first advanced by Metchnikoff that antibodies originated in the leucocytes failed of experimental proof. During the past few years particular attention has centered about the spleen, lymph nodes, and bone marrow as important organs of hemolysin formation. Hektoen[1] showed that splenectomized rats developed hemolysin to a less degree than the normal controls. This did not hold true for the rabbit. Kyes[2] called attention to the fact that foreign red cells introduced into the circulation are destroyed by the endothelial phagocytes of the liver and spleen. Cary,[3] impressed by Kyes' observations, developed the hypothesis, from a series of experiments, that the splenic endothelial cells which phagocyted the foreign red cells produced the hemolysin. He based his conclusions on evidence that hemolysin was much more concentrated in the spleen than in the liver or serum of immunized rabbits. Motohashi[4] showed that the physiological hemophagic activity in the rabbit is confined to the spleen. After splenectomy, however, there occurs after a certain period, a compensatory hemophagic activity in the bone marrow and liver. He was able to show that rabbits injected 2 or 3 weeks after splenectomy, or during the period when the compensatory hemophagic activity was at a minimum, developed hemolysin to a much less degree than the controls.

Experiments dealing with the formation of bacterial antibodies are on the whole less convincing. Wassermann and Citron[5] determined that lysin was more concentrated in the liquid portions of the exudates produced by intraperitoneal or intrapleural injection of typhoid bacilli than it was in the blood serum. It was suggested that the lysin was made by the cells of the cavities. Hektoen[6] was unable to confirm this in the case of dogs injected with foreign

[1] Hektoen, L., *J. Infect. Dis.*, 1920, xxvii, 23.

[2] Kyes, P., *Internat. Monatschr. Anat. u. Physiol.*, 1914–15, xxxi, 543.

[3] Cary, W. E., *J. Med. Research*, 1922, xliii, 399.

[4] Motohashi, S., *J. Med. Research*, 1922, xliii, 419; 473.

[5] Wassermann, A., and Citron, J., *Z. Hyg. u. Infectionskrankh.*, 1905, i, 331.

[6] Hektoen, L., *J. Infect. Dis.*, 1911, ix, 103.

red cells. Pfeiffer and Marx,[7] Wassermann,[8] and Castellani[9] all showed that bacteriolytic substances were more concentrated in the spleen, bone marrow, and lymph nodes early in immunization than they were in the blood serum.

Isolated experiments, however, indicate that antibody may be produced by other tissues. One of Wassermann and Citron's observations suggests that the tissues surrounding the site of inoculation may produce antibody to a certain extent. Zinsser[10] cites the findings of Römer[11] who instilled abrin into a rabbit's eye and found that the retina developed strong antitoxic properties. The retina of the untreated eye remained inactive. Theobald Smith, Orcutt, and Little[12] have shown that the milk drawn from a quarter infected with *B. abortus* contains agglutinin in higher concentration than milk from uninfected quarters. As a result of a series of experiments in which various quarters were inoculated with living or dead cultures of *B. abortus* they concluded that there is a distinct participation of the udder in the production of agglutinin. Recently Murphy and Sturm[13] have shown that there is an apparent relationship of the lymphoid structure to the production of antibody. They showed that x-rays in doses sufficient to deplete lymphoid tissues seriously interfered with the production of precipitin and agglutinin. Dry heat, shown by them to stimulate the activity of lymphoid structures, leads to the production of antibody in greater concentration.

Inasmuch as most of the previous studies dealt with antibody other than agglutinin it seemed desirable to study other possible points of origin than those already mentioned. A procedure which did not affect the general health of the animal or alter the normal metabolic processes was particularly desirable. With these points in view the following methods were adopted.

Methods.

It seemed probable that extracts could be prepared from dried material. With such a procedure more accurate comparisons would be possible, since tissues rich in liquids could be compared with those of low water content on the basis of total dry matter. It will be shown

[7] Pfeiffer, R., and Marx, *Z. Hyg. u. Infectionskrankh.*, 1898, xxvii, 272.
[8] Wassermann, A., *Berl. klin. Woch.*, 1898, xxxv, 209.
[9] Castellani, A., *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvii, 381.
[10] Zinsser, H., Infection and resistance, New York, 3rd edition, 1923.
[11] Römer, cited by Zinsser.[10]
[12] Smith, T., Orcutt, M. L., and Little, R. B., *J. Exp. Med.*, 1923, xxxvii, 153.
[13] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1925, xli, 245.

that desiccated material when extracted with distilled water admirably meets these requirements.

The vaccine employed was of standard turbidity. It was prepared by suspending the growth from 24 hour slant agar cultures of the hog cholera bacillus in salt solution. The suspension was heated at 60°C. for 1 hour.

The rabbits, of approximately the same age and weight, were injected intravenously. After varying intervals they were stunned and the neck vessels severed, according to the procedure used in an abattoir. In this way the tissues were freed from considerable blood. As rapidly as possible the organs were removed and ground in mortars. The tissues were then spread in small Petri dishes in thin layers and dried *in vacuo* over sulfuric acid. All tissues were completely desiccated within 24 hours. Structures rich in lipoids, like the bone marrow and lymph nodes, remained greasy, often of the consistency of butter. The desiccated material was ground into powder and stored in tightly stoppered tubes. For use it was weighed and suspended in distilled water in the proportion of 0.2 gm. of powder to 4 cc. of water. This proportion was finally chosen as a standard after several trials. It was found that this amount of dried rabbit serum would be dissolved in 4 cc. of distilled water. Extraction was carried out in tightly stoppered tubes for 4 hours at 36°C. All tubes were shaken at 15 minute intervals. They were then stored in the refrigerator for 16 or 18 hours and again shaken and centrifuged. The supernatant liquid varied in color and opacity. It was tested for agglutinin content against salt solution suspensions of living antigen.

The process of desiccation and extraction described might conceivably injure the antibody or so change it as to render it insoluble. To test this point the following experiment was performed.

Experiment 1.—3 cc. of the serum of an immunized rabbit, rich in agglutinin for the hog cholera bacillus, was dried over sulfuric acid *in vacuo*. 0.202 gm. of the dried material was obtained. 0.2 gm. of this was extracted with 4 cc. of distilled water in the manner described. As a control, 1 cc. of distilled water was added to 3 cc. of the immune serum. Both were tested for agglutinin content with a suspension of living hog cholera bacilli. The results of the comparative tests are given in Table I.

It will be noted that the agglutinin is not quite as strong in the extract of dried serum as in the serum unchanged except for the addition of distilled water. The experiment, however, indicates that the antibodies were not seriously injured during the process of desiccation. It is further indicated that the extraction of the dried serum with distilled water is efficacious. The method then was sufficiently

TABLE I.

A Comparison of the Agglutinin Titer of Dried Immune Serum and That of Serum Diluted to the Same Concentration with Distilled Water.

Materials tested.	Dilutions.						
	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	1:10,000
Extract of dried serum.....	C.*	C.	C.	++++	+++	++	++
Control serum diluted to 1:3 with distilled water.....	C.	C.	C.	C.	+++	+++	+++

* Throughout the tables, C. indicates a complete agglutination; the degree of precipitation is given in terms of +: ++++ indicates a strong agglutination, and the weaker are recorded as +++, ++, +.

TABLE II.

Native Agglutinins for the Hog Cholera Bacillus in the Extracts Prepared from Blood, Serum, and Organs of Normal Rabbits.

Rabbit No.	Extracts tested.	Dilutions of extracts.				
		1:5	1:10	1:20	1:50	1:100
1	Blood.	+	±	−	−	−
2	"					
1	Serum.	++	+	+	−	−
2	"	++	+	−	−	−
1	Lung.	±	−	−	−	−
2	"	±	−	−	−	−
1	Liver.	++	+	−	−	−
2	"	++	+	−	−	−
1	Spleen.	±	−	−	−	−
2	"	±	−	−	−	−
1	Kidney.	±	−	−	−	−
2	"	−	−	−	−	−
1	Lymph glands.	±	−	−	−	−
2	" "	+	−	−	−	−
1	Bone marrow.	−	−	−	−	−
2	" "	−	−	−	−	−

accurate for further experimentation. In the experiments the extracts were prepared usually within a week. The material was retested again after storage for 2 weeks at room temperature. A few tests made on material stored at room temperature over the summer showed that the serum, liver, and other organs were no longer as soluble as when freshly dried.

The next step was to establish the agglutinin content of various organs of normal animals.

Experiment 2.—On different occasions normal rabbits were killed and bled in the manner described. Portions of blood, serum, lungs, liver, spleen, kidneys, lymph glands, and bone marrow were dried *in vacuo* over sulfuric acid. Aqueous extracts were prepared and tested for natural agglutinin with salt solution suspensions of the hog cholera bacillus. It was found that the excess of hemoglobin of whole blood prevented accurate readings and its use was therefore discontinued. The results obtained from two normal rabbits are given in Table II.

The results of a number of examinations of normal animals have been that the natural agglutinin is always present in the serum at a slightly higher concentration than in the organs. Of the organs the liver is richest in antibody, but in the normal individual its concentration is slight.

Having established the normal agglutinin content of the various organs the next step undertaken was to ascertain if possible in what organs the greatest concentration of agglutinin occurred during immunization. At first I attempted to establish this by intravenous injections of several small doses of vaccine, permitting several days to elapse between the last injection and examination of the tissues. It was found, however, that with such a procedure the antibody was more concentrated in the serum than in the tissues. The details of a single observation illustrating this fact are given in Experiment 3.

Experiment 3.—Rabbit 3 was injected intravenously on 3 successive days with six small doses of vaccine. The reactions following each injection were severe. The amount of agglutinin in the blood was followed from day to day. 5 days after the last injection the animal was killed. The serum and organ extracts were prepared in the usual way. The results are given in Table III.

In the highly immunized animal the antibody is at its greatest concentration in the blood serum, as the table indicates. Hektoen and

Carlson,[14] however, have clearly shown that the blood itself is incapable of producing antibody. Therefore it is possible that the period between the last injection of antigen and the tests of the organs was of sufficient length to permit the passage into the blood stream of the major portion of antibody from the site of its formation. The results of the tests of certain organs, however, were suggestive. The liver and kidneys possessed the greatest concentration of antibody; there was less in the spleen and lymph glands. With these facts established it was decided to continue immunization along the same lines but to inject smaller doses of vaccine and to decrease the interval between the last injection and the death of the animal.

TABLE III.

The Agglutinin Content of the Extracts of Dried Serum and Organs of a Rabbit Immunized by Multiple Doses of Vaccine.

Extracts tested.	Dilutions of extracts.							
	1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000
Serum	C.	C.	C.	+++	++	++	+	±
Lung	++	+	+	±	−	−	−	−
Liver	C.	+++	++	+	+	+	+	±
Spleen	+++	++	++	+	+	±	−	−
Kidney	C.	+++	+	+	+	+	±	−
Lymph gland	C.	++	+	+	+	±	−	−
Bone marrow	++	++	+	+	±	−	−	−
Testicle	+	+	±	−	−	−	−	−
Muscle	+++	+	±	−	−	−	−	−
Small intestine	+++	+	+	±	−	−	−	−

The following may be regarded as a type experiment. The protocols of two rabbits only are submitted. Other rabbits treated in the same manner gave similar results.

Experiment 4.—Rabbit 4. Bled before injection. Its serum when tested only slightly clumped the bacilli at a dilution of 1:5. The animal in the course of 2 days received three intravenous injections aggregating 0.85 cc. of vaccine. Moderate temperature reactions followed each injection. 25 hours after the last injection the animal was killed.

[14] Hektoen, L., and Carlson, A. J., *J. Infect. Dis.*, 1910, vii, 319.

Rabbit 5. The blood serum obtained before injection failed to agglutinate the antigen at a dilution of 1:5. Four intravenous injections of vaccine aggregating 1.85 cc. were made during 2 days. The animal was killed 48 hours after the last injection.

The results of the tests are recorded in Table IV.

TABLE IV.

The Agglutinin Content of the Dried Serum and Organs of Rabbits Immunized by Intravenous Injections and Killed 1 and 2 Days after the Last Injection.

Rabbit No.	Extracts tested.	Dilutions of extracts.					
		1:5	1:10	1:20	1:50	1:100	1:200
4	Serum.	++	+	−	−	−	−
5	"	++	+	±	±	−	−
4	Lung.	++	+	+	±	−	−
5	"	+++	++	+	±	−	−
4	Liver.	++++	+++	++	+	±	±
5	"	++++	++++	+++	++	+	±
4	Spleen.	+	±	±	−	−	−
5	"	+++	++	+	±	−	−
4	Kidney.	+++	++	+	±	−	−
5	"	+++	++	+	±	−	−
4	Lymph gland.	+	−	−	−	−	−
5	" "	++	+	+	±	−	−
4	Bone marrow.	+	±	−	−	−	−
5	" "	+	+	±	±	−	−

The results of the tests indicate that early in the period of immunization agglutinin is present in greater concentration in certain organs than it is in the serum. The liver contains agglutinin in the greatest concentration under these conditions. The lungs and kidneys contain, as a rule, more than the spleen, lymph glands, and bone marrow. The inference then that antibody is produced to a considerable extent in several organs, especially the liver, seems reasonable. Several explanations of the high concentration of antibodies in the liver must be considered. Considerable blood may have remained in the organ

but only 50 or 60 per cent of this could have been serum, since the titer of the serum is considerably below that of the liver extract. Another point that might be raised is the possibility of the liver as a storage place for antibodies. The results given in Table III indicate, however, that when the serum of a highly immunized individual is rich in antibody the liver contains considerably less. It seemed possible to definitely settle this question by the following experiment.

Experiment 5.—Rabbit 6. Injected intravenously with 4 cc. of immune rabbit serum. 1/2,000 cc. of the recipient's serum obtained shortly after the injection would clump the bacilli. The animal was bled at daily intervals. At the end of 7 days the concentration of agglutinin in the circulating blood had markedly declined and the animal was killed. Portions of the serum and organs were desiccated and extracted. The extracts when tested for agglutinin gave the results recorded in Table V.

TABLE V.

The Concentration of Agglutinin in the Serum and Tissues of a Passively Immunized Rabbit.

Extracts tested.	Dilutions of extracts.					
	1:10	1:20	1:50	1:100	1:200	1:500
Serum	C.	+++	++	+	±	−
Lungs	++++	++	±	−	−	−
Liver	++	+	+	−	−	−
Spleen	+	±	−	−	−	−
Kidneys	+	+	±	−	−	−
Lymph gland	+	+	±	−	−	−
Bone marrow	±	±	−	−	−	−
Muscle	+	±	−	−	−	−

Similar results were obtained when another rabbit was treated in the same manner. This experiment amplifies Experiment 2 and indicates that the liver is not a storage place for agglutinin since it persists in the blood in greater concentration than in any of the organs. It has long been known that in passive immunity antibodies disappear from the blood. The assumption has been that they are eliminated from the body. This explanation would seem to be the correct one, since they are not stored in the organs.

A final series of experiments seemed desirable. Evans, Bowman,

and Winternitz[15] showed that dead tubercle bacilli injected into a radicle of the mesenteric vein were transported to the liver and ingested by Kupffer cells. By the use of a similar technique it was hoped that most of the antigen would first come in contact with the liver and there be utilized, thus giving rise to a greater concentration of agglutinin within the organ. While this proved to be not wholly the case the results indicate that in the main it was true. An outline of the procedure and findings is given in the following experiment.

Experiment 6.—Rabbit 7. Anesthetized with ether. A median line incision was made through the skin and tissues which comprise the abdominal wall.

TABLE VI.

The Concentration of Agglutinin in the Serum and Organs Subsequent to Injection of Antigen into a Radicle of the Mesenteric Vein.

Extracts tested.	Dilutions of extracts.					
	1:5	1:10	1:20	1:50	1:100	1:200
Serum	+	±	−	−	−	−
Lungs	−	−	−	−	−	−
Liver	C.	C.	+++	+	+	±
Spleen	+	+	±	−	−	−
Kidneys	+	+	±	−	−	−
Ascitic fluid	+	+	±	−	−	−
Bone marrow	±	−	−	−	−	−
Small intestine*	+++	+	−	−	−	−
Lymph gland	+	±	−	−	−	−

* Includes the mesentery surrounding the site of injection.

With rubber covered forceps a loop of small intestine was withdrawn through the opening and a fine hypodermic needle introduced into a branch of the mesenteric vein and 0.75 cc. of standard vaccine together with 1.25 cc. of salt solution slowly injected. The incision was closed with silk and a suspensory bandage applied. The animal made an uneventful recovery. 4 days later it was killed and the serum and tissues desiccated. Autopsy failed to show gross changes in the viscera. There was a little clear fluid in the abdominal cavity. The wound was uninfected and healing processes well advanced. The results of tests of the extracts are shown in Table VI.

[15] Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.

Another rabbit killed 3 days after a smaller injection into a branch of the mesenteric vein also developed a concentration of agglutinin in the liver ten times as great as that of the blood serum and five times greater than in the spleen. In these instances presumably most of the antigen first came in contact with the liver. It seems probable that it was utilized within this organ.

A number of other experiments were performed in which rabbits were injected intratracheally, intramuscularly, and intratesticularly. In certain instances the concentration of agglutinin was slightly greater in the liver than in the other organs and serum, but the results were not as definite as those reported when the injections were made intravenously.

The method of drying and extraction seemed well adapted to the study of the local formation of antibodies. For the purpose rabbits were injected intramuscularly and intratesticularly with vaccine, and in certain instances the site of injection was first prepared by the administration of kieselguhr or agar. In no instance was it possible to show that the injected muscle or testicle possessed more antibody than the corresponding uninjected areas.

DISCUSSION.

Of considerable interest are the findings of others concerning the destruction of bacteria introduced into the blood stream.

Werigo[16] pointed out that anthrax bacilli injected intravenously were disposed of by the hepatic endothelium. Adami, Abbott, and Nicholson[17] injected colon bacilli into the veins of young rabbits and killed them at intervals. They noted that within a short time the endothelial cells lining the capillaries of the liver began to phagocyte the bacilli. Within 2 hours the endothelial cells contained large numbers of bacilli and no free bacilli remained in the capillaries. They pointed out that certain cells of the kidney possessed the same faculty but to a less degree. Evans, Bowman, and Winternitz, whose experiments have been previously referred to, were able to show that dried tubercle bacilli when they reached the liver were taken up by Kupffer cells. Kyes[18] in studying the natural resistance of the pigeon to the pneumococcus encountered the same phenom-

[16] Werigo, *Ann. Inst. Pasteur*, 1894, viii, 1.

[17] Adami, J. G., Abbott, M. E., and Nicholson, F. J., *J. Exp. Med.*, 1899, iv, 349.

[18] Kyes, P., *J. Infect. Dis.*, 1916, xviii, 277.

enon. He pointed out that the cocci were rapidly taken from the liver circulation by the hemophages lining the capillaries. He also stated that the hemophages from the spleen contained as many cocci cell for cell as those of the liver, but in the spleen such cells are much less numerous than in the liver. Certain specific peculiarities of the anatomical structure of the walls of the smaller splenic vessels of the pigeon permit the intravascular accumulation of pneumococci, greatly facilitating phagocytosis. Bull[19] noted that typhoid bacilli were destroyed by phagocytes in the liver and spleen of rabbits which had received large doses of culture intravenously. He considered the polymorphonuclear leucocytes of prime importance in the process.

It will be seen that there is considerable accord concerning the importance of the liver as a locus of accumulation and destruction of bacteria entering the blood stream. With the small doses of dead bacteria employed in my experiments it seems entirely possible that a great bulk of the bacteria were destroyed by the liver and that the agglutinin was formed there during the process. The importance of the endothelial cells lining the capillaries as active phagocytes of bacteria and other substances suggests these cells as a possible source of antibody formation. The fact that the "plugging" of endothelial phagocytes with trypan blue inhibits antibody production, as recorded by Gay and Clark,[20] strengthens such a view.

It is usually conceded that antigens injected intravenously lead to a more rapid and usually to a greater production of antibodies than injections by other routes. It is also true that the reactions occurring from such a procedure are usually more severe. Under such conditions much of the antigen, particularly bacterial cells, may accumulate in the liver where there exists ample opportunity for rapid phagocytosis and consequent destruction. Antigen injected by routes other than the blood stream would reach the liver and other organs more slowly and the resulting reactions would be milder in character and perhaps lead to a slower increase in antibodies. It should be emphasized that in the opinion of the writer the liver is not the only organ of importance in agglutinin formation. Doubtless agglutinins are formed in many structures. The work of Theobald Smith, Orcutt, and Little indicates that the udder plays a part in

[19] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 475.

[20] Gay, F. P., and Clark, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 1.

agglutinin production. The experiments indicate, however, that bacterial antigen introduced into the systemic or portal circulation leads to a considerable increase of antibody within the liver.

SUMMARY.

Serum and tissues containing agglutinin for the hog cholera bacillus may be dried *in vacuo* over sulfuric acid without appreciably injuring the antibody. The desiccated material when extracted with appropriate amounts of distilled water offers a basis for accurate comparison of antibody content. The greatest concentration of agglutinin occurred in the liver, provided the animals injected with small amounts of antigen were killed within a short period. The serum of those more highly immunized contained the greatest concentration of antibody. A single injection of antigen into a radicle of the mesenteric vein resulted in a considerable concentration of agglutinin in the liver. Other experiments indicated that the liver does not act as a reservoir for the antibody. It has also been shown that this concentration of agglutinin cannot be ascribed to the blood left within the liver, since the blood serum was relatively poor in antibody. The experiments indicate that the agglutinin was produced within the liver.

SURFACE TENSION OF SERUM.

XIII. On Certain Physicochemical Changes in Serum as a Result of Immunization.

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Plates 25 and 26.

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The late changes in the static surface tension of serum solutions following antigen injections in rabbits, have been studied in two papers of this series, and it has been shown that the "time-drop" (difference between the initial value of the surface tension of the solution and its value after standing 2 hours in a watch-glass) increases in parallel with the antibody content.[1] Curves have been published exhibiting the progressive increase of the time-drop after a certain period, and the establishment of a maximum value between the 12th and 13th days after the first injection. In the experiments, the animals (rabbits) were injected with various proteins and with smallpox vaccine, but were always given three successive injections or more, except in the case of vaccine. The question arose as to whether the same phenomena would occur, if the animals received but a single injection of antigen; also whether the maximum would occur after the same number of days, and be of the same magnitude; and whether the amplitude of the drop would be a function of the amount of antigen injected. The further question was raised whether a new injection, just after the peak of the time-drop had been reached (13th day), would result in an exaggeration of the phenomenon, or delay its decrease.

EXPERIMENTAL.

Twelve rabbits were used, six as controls. All these animals were studied for a period of 2 months before the injection, in order to make

[1] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 659.

certain that they had normal time-drops, that is to say time-drops of less than 10 dynes.[1,2] When this had been ascertained, they were injected intravenously with the antigen made from crushed horse kidney[3] and provided by Dr. Landsteiner. This antigen was chosen because of its harmlessness and its constant results. In previous work, some trouble had been experienced with other antigens. One of the animals died just before the experiment was started, so only five were left, in addition to the controls. Rabbit 1 received 4 cc. of the antigen; Rabbits 2 and 3, 5 cc.; Rabbits 5 and 6, 10 cc. All were, of course, kept and fed in the same way as the controls. Measurements were made every other day. In the following table, crosses indicate the time of the bleedings.

TABLE I.

Day after antigen injection.		5	6	7	8	9	12	13	15	16	19	20	21	23	26	27	28
Controls.	No. 1	×		×		×	×	×		×		×		×		×	
	" 2	×		×		×	×	×		×		×		×		×	
	" 3	×		×		×	×	×		×		×		×		×	
	" 4		×		×		×	×	×		×		×		×		×
	" 5		×		×		×	×	×		×		×		×		×
	" 6		×		×		×	×	×		×		×		×		×
Experimental.	No. 7	×		×		×	×	×		×		×		×		×	
	" 8	×		×		×	×	×		×		×		×		×	
	" 9		×		×		×	×	×		×		×		×		×
	" 10		×		×		×	×	×		×		×		×		×
	" 11		×		×		×	×	×		×		×		×		×

It will be seen that the controls and the experimental animals were bled alternately, except on the 12th and 13th days when the time-drop phenomenon was expected to reach its maximum. The reasons for not making the measurements every day were, first that the animals might be disturbed by too frequent bleedings, and, second because of technical difficulties. It must be borne in mind that seventeen different dilutions of the serum were made in every case. This in itself

[2] du Noüy, P. L., *J. Exp. Med.*, 1924, xl, 129.

[3] The horse kidney is crushed and diluted to 1:10 with physiological solution (NaCl, 0.9 per cent). 0.5 per cent of phenol is added as preservative. The mixture is allowed to settle and the supernatant fluid is used.

CORRECTION.

In Vol. xli, No. 6, page 781, lines 22 to 25 should read: ". . . . and each step of 500 in the dilution to a difference of 7,000 in the proteins. This in turn corresponds to a difference in thickness of the adsorbed layer of, roughly, 2.2×10^{-8} cm. between 1:10,000 and 1:11,000."

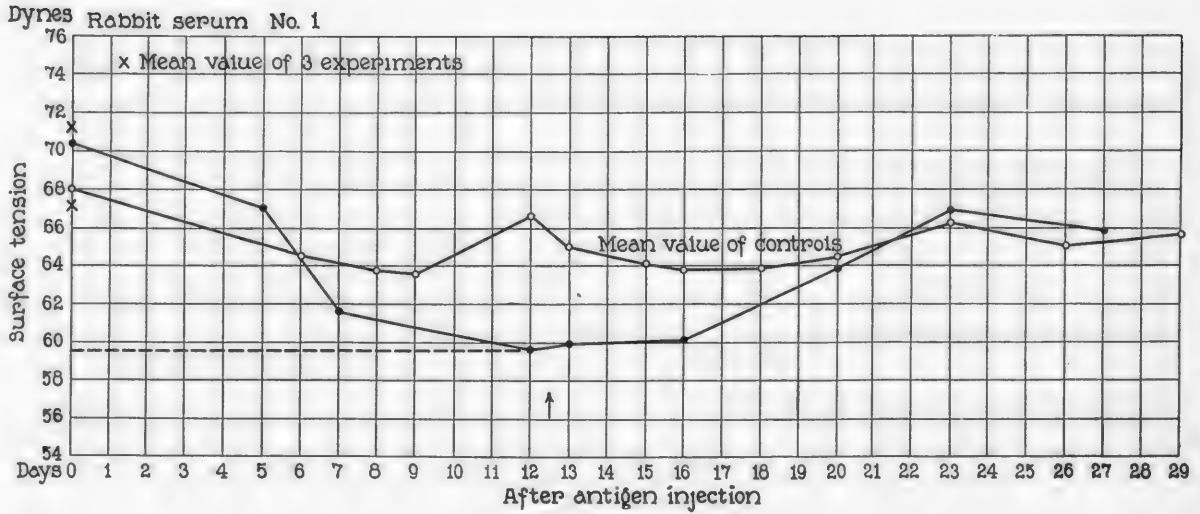
required considerable glassware which had to be cleaned with the utmost care. As two measurements of the surface tension were required, 2 hours apart, this meant thirty-four measurements for each serum. When all sera were dealt with, as on the 12th and 13th days, 374 measurements of surface tension had to be made in 1 day. This amount of labor can be carried out during 2 days, but it becomes extremely taxing on all concerned when done oftener. Fortunately this appeared unnecessary. As matters stand, the first set of experiments reported in the present paper required a total sum of 4,420 measurements of surface tension. They were rendered possible through the simultaneous use of three tensiometers on tables of a sort previously described,[4] each table holding forty-four watch-glasses.

The seventeen different dilutions of every serum were made because it was important to know whether the maximum drop, or absolute minimum of static surface tension always occurred at the same dilution, or whether it was slightly shifted, perhaps from an unknown cause, perhaps as a consequence of the injection of antigen. The following dilutions were studied: 1:10, 1:100, 1:1,000, 1:7,500, 1:8,000, 1:8,500, 1:9,000, 1:9,500, 1:10,000, 1:10,500, 1:11,000, 1:11,500, 1:12,000, 1:12,500, 1:13,000, 1:100,000, and 1:1,000,000. The 1:7,500 and 1:13,000 dilutions correspond approximately to dilutions of the proteins of 1:105,000 and 1:182, 000 respectively, and each step of 1:500 in the dilution to a difference of 1:7,000 in the proteins. This in turn corresponds to a difference in thickness of the adsorbed layer of, roughly, 2.2×10^{-8} cm. As the thickness of the monolayer of rabbit serum is about 41×10^{-8} cm.,[5] this means that a change of about 5 per cent in the thickness could be detected. On taking into account the working conditions, and the variations likely to arise from changes in the concentration of the proteins over a period of 3 months in the animal life, this was considered as the maximum of accuracy that could be reasonably expected.

Table II gives the concentrations at which the minimum surface tension was observed for the five experimental animals and two controls. It is obvious that no constant shift occurs after immunization. Such differences as were noted may be laid to experimental errors.

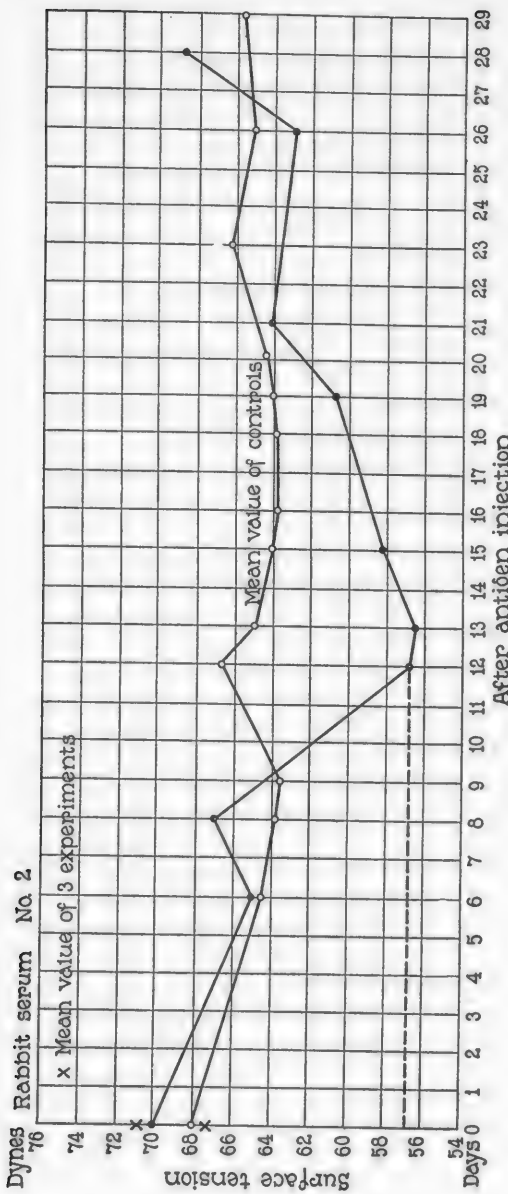
[4] du Noüy, P. L., *J. Gen. Physiol.*, 1923–24, vi, 625.

[5] du Noüy, P. L., *J. Exp. Med.*, 1924, xl, 133.

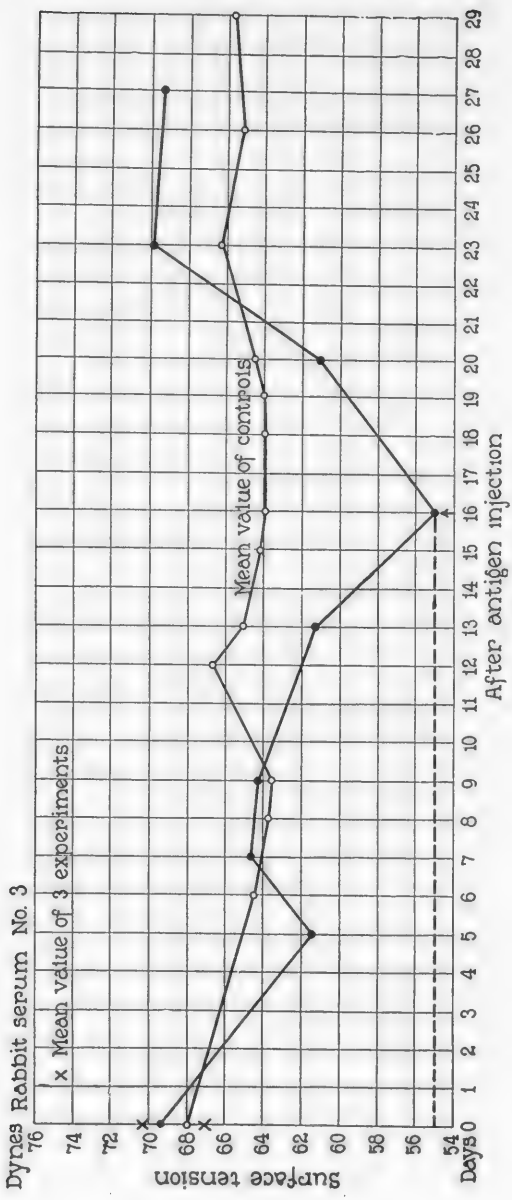


TEXT-FIG. 1.

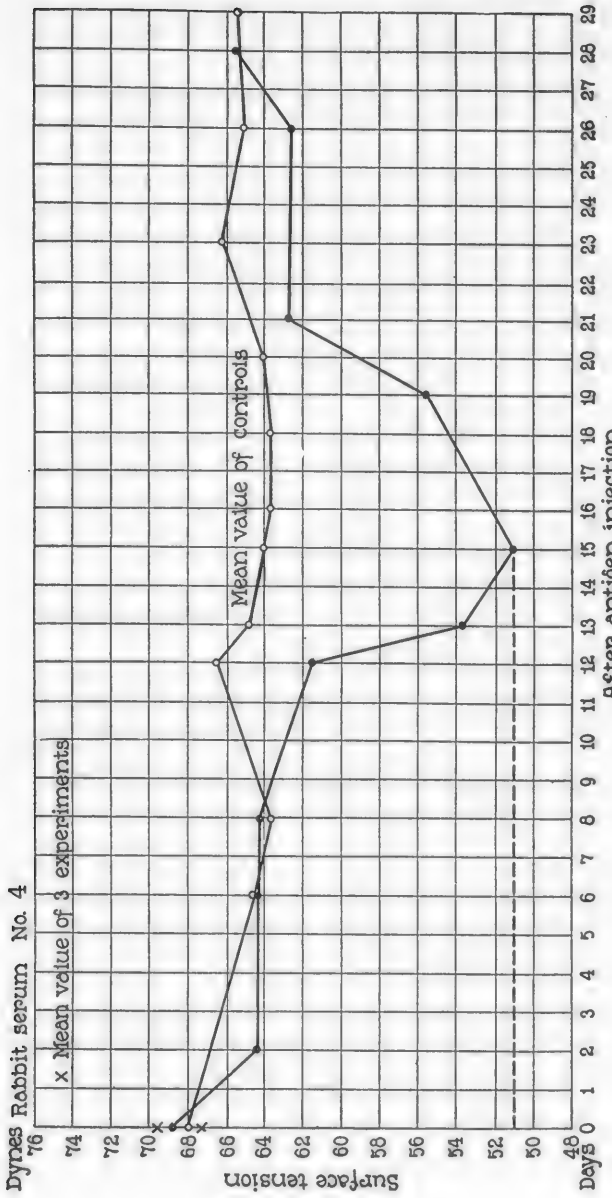
TEXT-FIGS. 1 to 5. Static values, after 2 hours, of immune rabbit serum diluted to the degree corresponding to the monolayer, in function of time, in days. The upper curve is the mean value of 2 controls.


Dynes
Rabbit serum No. 2
× Mean value of 3 experiments
Mean value of controls
Surface tension
After antigen injection
Days
76
74
72
70
68
66
64
62
60
58
56
54
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

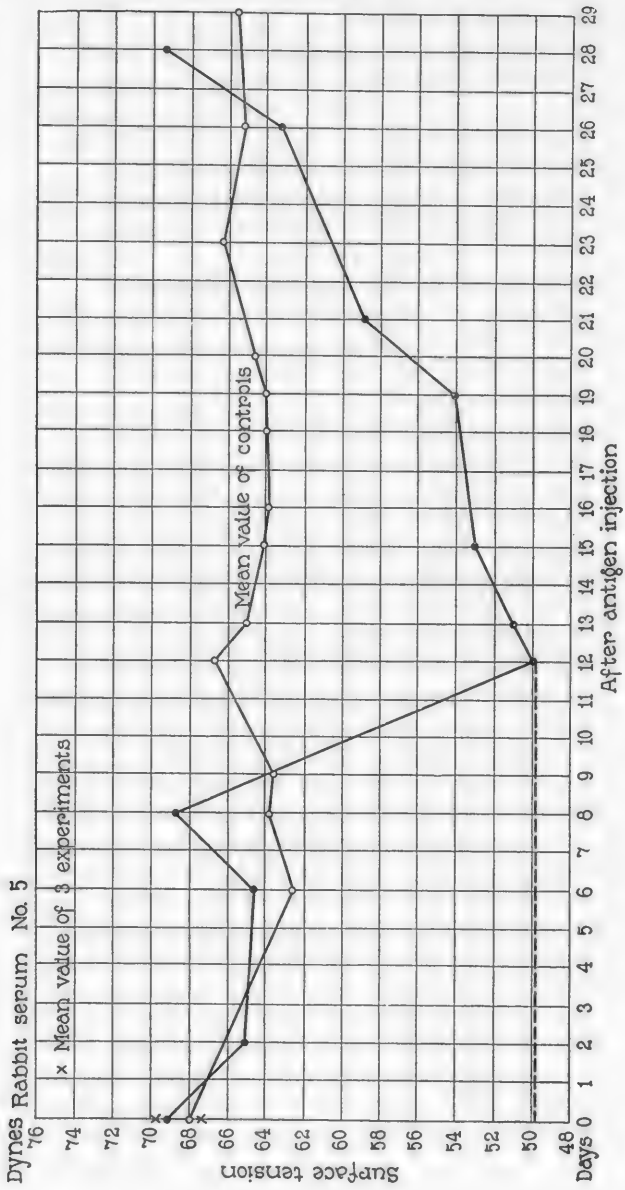
TEXT-FIG. 2.


Dynes Rabbit serum No. 3
× Mean value of 3 experiments
Mean value of controls
Surface tension
76
74
72
70
68
66
64
62
60
58
56
54
Days 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
After antigen injection

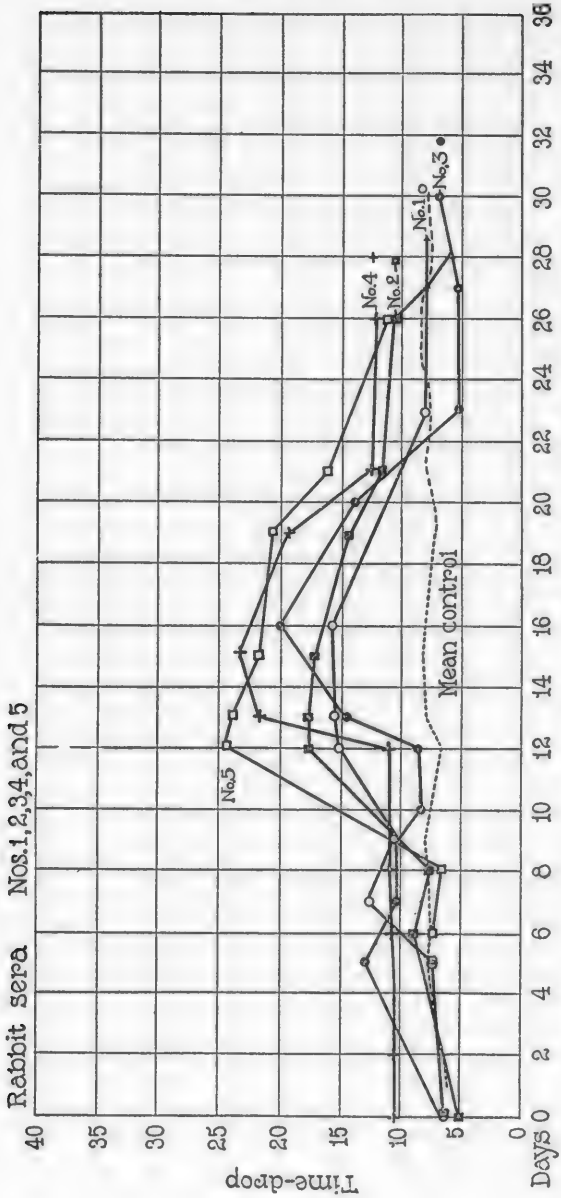
TEXT-FIG. 3.


Dynes Rabbit serum No. 4
x Mean value of 3 experiments
Mean value of controls
Surface tension
After antigen injection
Days 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
76 74 72 70 68 66 64 62 60 58 56 54 52 50 48

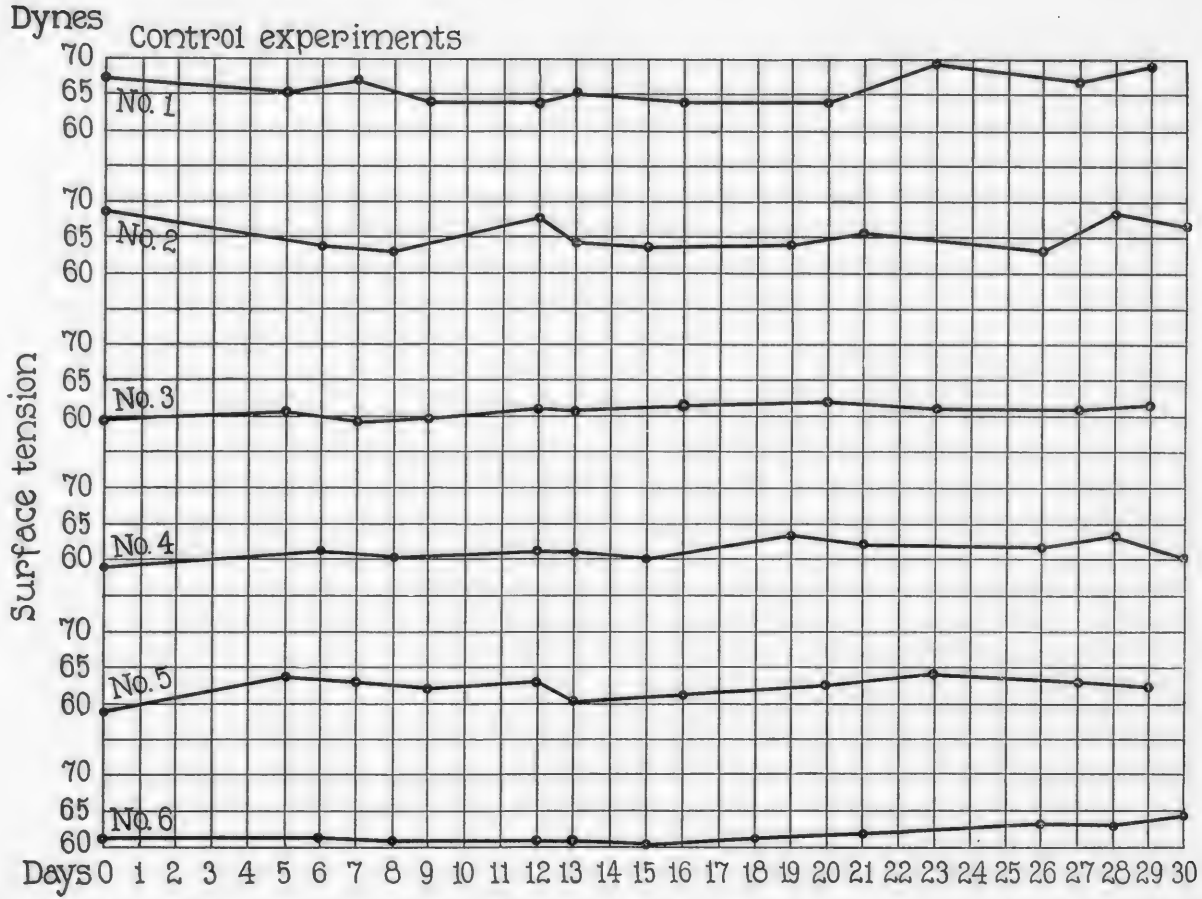
TEXT-FIG. 4.


Dynes
Rabbit serum No. 5
× Mean value of 3 experiments
Mean value of controls
Surface tension
Days 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
After antigen injection
76 74 72 70 68 66 64 62 60 58 56 54 52 50 48

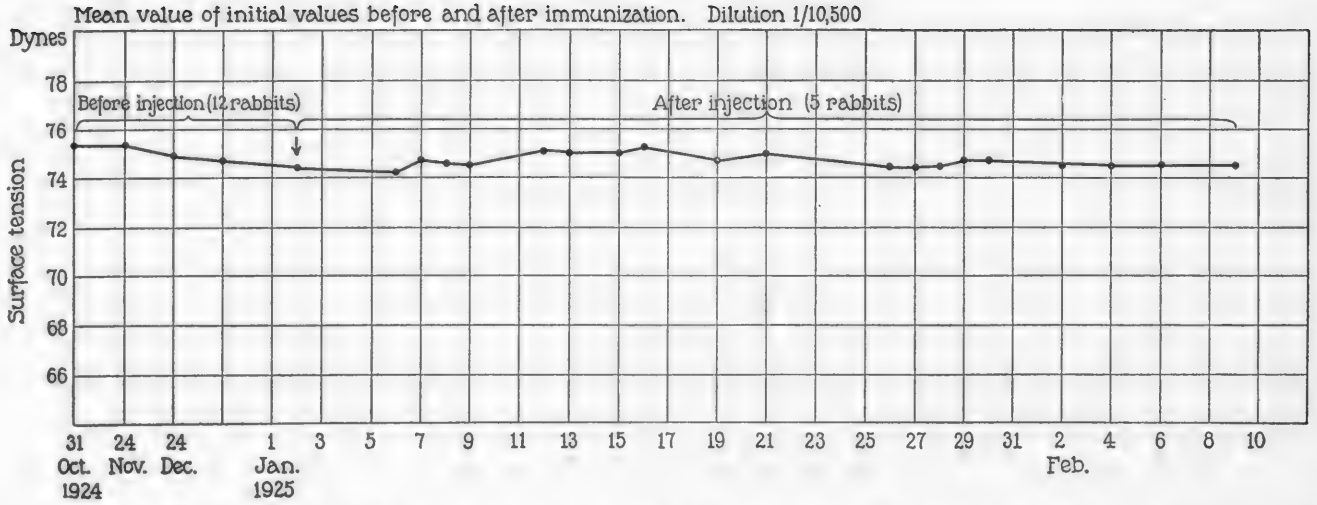
TEXT-FIG. 5.



TEXT-FIG. 6. Time-drop, after 2 hours, of sera Nos. 1, 2, 3, 4, and 5, and control curve (6 controls).



TEXT-FIG. 7. Static values, after 2 hours, of normal rabbit sera (controls), in the same conditions as the experiments reported on Text-figs. 1 to 5.



TEXT-FIG. 8. Mean value of initial surface tension, before and after immunization. The figure shows that the increased time-drop is due to an actual decrease in the static value of the surface tension, and not to a change in the initial value.

only at a very definite concentration, namely that which corresponds to the absolute minimum value of the static surface tension.[6] Fig. 2 is a photomicrograph taken with polarized light, of a control, while Fig. 3 is a picture taken under the same conditions, of immune serum No. 8. The difference in structure will be evident.

DISCUSSION.

In the light of the experiments described above, it can be stated that an important reaction takes place in the circulation of the animals subjected to injections with certain antigens. This reaction begins to affect the field of forces of the molecules of some of the protein constituents of the serum about 8 days after the injection. The disturbance resulting from this reaction reaches a maximum some time between the 12th and the 16th days, generally between the 12th and 13th days. Then it decreases, and finally disappears around the 30th day. At the time of the maximum, a considerable number of molecules are involved, as shown by the striking effect on the crystallization of NaCl. The evolution of this phenomenon, when the time-drop is plotted in ordinates and the time of days in abscissæ, follows closely the curves obtained by Jörgensen and Madsen[7] (immunity reaction in the test-tube) and by Fischer.[8]

The phenomenon cannot be due to the mere presence of antigen in the circulation for the following reasons. First, the maximum always occurs around the 13th day, no matter whether the antigen has been injected in a massive dose on the 1st day, or whether it has been injected in four successive doses on the 1st, 5th, 8th, and 11th days.[1] Second, when an amount of antigen equal to the initial quantity is injected into the animal the day after the maximum time-drop has been reached, the evolution of the phenomenon is not affected and no further increase of the time-drop is observed. The latter goes on

[6] However, it does not seem to occur in all cases, as a few further experiments did not show it regularly.

[7] Jörgensen, A., and Madsen, T., Festkrift ved Indvielsen af Statens Serum Institut, in Contributions from the University Laboratory for Medical Bacteriology to celebrate the inauguration of the State Serum Institute, Copenhagen, 1902, Paper 6, 12.

[8] Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 535.

decreasing gradually until the 30th day. Third, animals reinjected at the end of 30 days fail to show the increased time-drop again.

CONCLUSIONS.

1. The injection of antigen into an animal determines a gradual change in the blood fluid which finds expression in two physicochemical manifestations that can readily be followed, namely a decrease in the static value of the surface tension of serum solutions, and a special form of crystallization when serum diluted with isotonic sodium chloride solution is allowed to evaporate under certain conditions.

2. The change in the blood is at a maximum around the 13th day after the first antigen injection, and decreases progressively thereafter until it can no longer be observed, which is usually around the 30th day.

3. The change follows the same course, whether a single large injection of antigen is made, or many smaller ones. It begins at the same time in either case, it comes to a maximum after the same period, and in its subsequent course it is not affected by the reinjection of antigen. The manifestations of the change would appear to be independent of the presence of antigen in the circulation.

4. The mean length of the protein molecules of the immune serum obtained after the injection of the antigen dealt with is little if at all different from that of the protein molecules of normal serum.

5. It is possible that this reaction is independent of the antibody formation.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. Photographs, slightly reduced, of watch-glasses after evaporation of the solution. The top row *a* is a normal serum (control). The two lower rows, *b* and *c*, are immune sera. In these two series the NaCl crystals at the dilution 1:10,500 are quite different from those of the normal serum, and also from those of other concentrations (1:10,000 and 1:11,000).

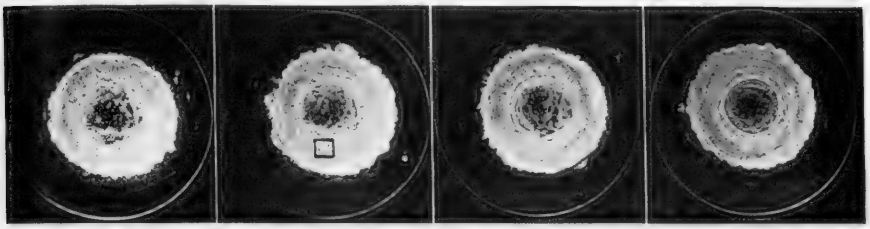
PLATE 26.

FIG. 2. A photomicrograph of the area delimited in black on the first row, *a* of Fig. 1 (concentration 1:10,500), normal serum. × 10.

FIG. 3. A photomicrograph of the area delimited in black on the last row, *c* of Fig. 1 (concentration 1:10,500), immune serum. × 10.

Both photomicrographs were both taken with polarized light, through a blue filter.

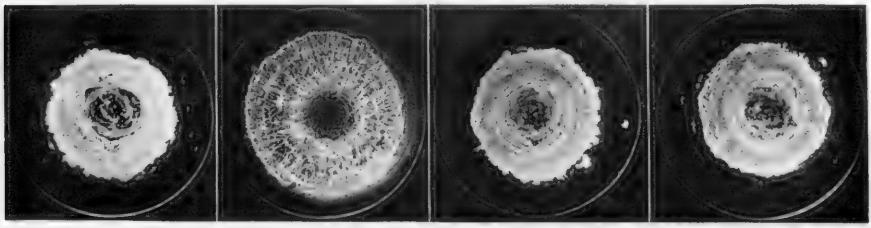
a



Dilution
of serum....1:10,000 1:10,500 1:11,000 1:11,500

Normal serum in 0.9 per cent NaCl solution (control).

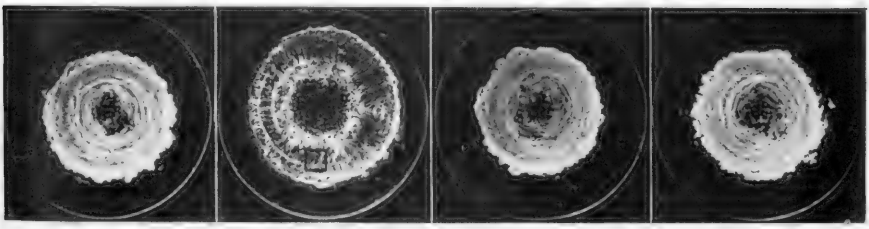
b



Dilution
of serum....1:10,000 1:10,500 1:11,000 1:11,500

Immune serum No. 7 in 0.9 per cent NaCl solution.

c



Dilution
of serum....1:10,000 1:10,500 1:11,000 1:11,500

Immune serum No. 8 in 0.9 per cent NaCl solution.

FIG. 1.

(du Noüy: Surface tension of serum. XIII.)

FIG. 2.

FIG. 3.

(du Noüy: Surface tension of serum. XIII.)

THE OCCURRENCE OF STIGMATA OF CONGENITAL SYPHILIS IN RABBITS WITH ESPECIAL REFERENCE TO THE FACTOR OF INHERITANCE.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 27 to 29.

(Received for publication, April 8, 1925.)

Syphilis in the rabbit is rarely, if ever, transmitted to the young *in utero* either by the mating of a normal with an infected animal or of one infected animal with another. It has been found, however, that the fertility of both sexes may be diminished to some extent by syphilis and that infection in the female may lead to abortion or premature birth with an increased proportion of still-births and a reduction in the proportion of viable young born at term. Malformations also appear to be more frequent. Evidence of infection *in utero* is very meager. Uhlenhuth and Mulzer (1) were able to recover spirochetes from fetal organs almost immediately after the intravenous injection of a large dose of organisms (10 cc. of a testicular emulsion rich in spirochetes) into a pregnant female with acutely fatal results, but experiments of this kind are of doubtful significance. These investigators also reported a single instance of an apparently valid transmission of infection by an animal inoculated in the same way at an early stage of pregnancy.[1] As far as we are aware, this is the only successful production of congenital infection in the rabbit that has been recorded.

As a rule, the young of syphilitic rabbits show no symptoms suggestive of syphilis; they develop normally, they are susceptible to inoculation with *Treponema pallidum*, and the few attempts that have been made to demonstrate infection in them by the inoculation of other animals have yielded negative results.

[1] Inoculation, Jan. 20, 1911. Litter of four young born Feb. 15, 1911; one of the young developed syphilitic lesions but no infection was demonstrated in the others.

In this laboratory we have noted a striking exception to the foregoing rule in a litter of young born of parents with a long standing syphilitic infection. At birth, these animals appeared to be normal, but within a short time they developed signs that were strongly suggestive of syphilis. The conditions which attracted our attention were a unilateral lesion of the cornea and iris and an abnormal growth of hair associated with an extensive alopecia which affected three of the six rabbits comprising the litter; but no infection could be demonstrated in any of these animals.

Our object in reporting the observations made on this group of animals is twofold. In the first place, the lesions and the circumstances of their occurrence are of interest in relation to so called stigmata of syphilis and to certain developmental peculiarities which have been attributed, by some, to a syphilitic ancestry rather than to actual infection of individuals who are the subjects of such conditions. The second object of the paper is to provide a background for subsequent reports on heredity based on a study of the progeny of the animals whose histories are given in this paper.

HISTORY.

Male Parent.—Young adult albino; weight at time of inoculation 1,400 gm.; maximum weight 1,700 to 1,800 gm.

On Dec. 10, 1918, the animal was inoculated in both scrota with the Zinsser-Hopkins strain of *T. pallidum*. In due time, a diffuse infiltration developed at both sites of inoculation and gradually spread over the surrounding skin with the formation of numerous small nodules distributed along the course of blood vessels and lymphatics. The primary lesions showed comparatively little activity, however, until about 2½ months after inoculation (late in February). At this time, secondary lesions began to appear in the skin, first over the outer sides of both hind feet, then over both forearms, the eye lids, and at the base of the ears. These lesions were unusually extensive and destructive and they remained active much longer than usual. Regression and healing did not begin until late in April. Meantime, the scrotal chancres had increased in size and number; they assumed the same destructive character as the secondary lesions and remained active for some time after the other lesions had healed.

For about a month, the animal was free from any active manifestation of syphilis but in June a relapse occurred and for the first time a distinct periosteal thickening of the nasal bones was detected. This relapse was of short duration and no other lesions were noted until late in September when a second relapse occurred. In this instance, the lesions were more numerous and more extensive.

From this time on (Sept., 1919) the animal was never free from syphilitic lesions. The course of disease was irregular and was marked by periods of regression followed by recrudescence or the appearance of new groups of lesions affecting the skin, bones, and eventually the eyes. It is worthy of note that the eyes did not become affected until Mar., 1921 or more than 2 years after inoculation and almost exactly 2 years after the appearance of the first generalized lesions. As a rule, the period of active syphilitic infection in the rabbit does not exceed 4 to 6 months but in this instance the disease remained active from the time of inoculation until death—a period of about 2½ years. In spite of this, the animal appeared to be in good physical condition for nearly 2 years. During this time he exhibited normal sexual activity and was used for breeding purposes in two experiments.

Between Oct. 8 and 15, 1919, the animal was mated with 3 infected females which may be designated as A, B, and C. A failed to become pregnant; B gave birth to a litter of 4 young, 3 of which survived and developed normally; C gave birth to 5 young, 1 of which was a still-born monster. This fetus showed malformation of the posterior extremities, absence of the tail and spleen, and an extreme grade of hydrocephalus with either an incomplete development or an almost total destruction of the brain. The 4 other members of this litter were poorly developed and lived for only a few days. The second mating of this rabbit was with Female A (Nov. 28, 1919) and from this mating came the litter of 6 young which are the subjects of the present report.

In Oct., 1920, the animal developed snuffles and there was a gradual physical deterioration interrupted by brief periods of improvement until June, 1921 when the condition of the animal became critical and he was anesthetized and killed for autopsy.

The significant features of the disease presented by this animal are: (1) the relatively slight initial response to infection; (2) the subsequent development of a malignant type of disease; and (3) an utter inability to cope with either the syphilitic infection or the intercurrent bacterial infection.

This is a typical example of a condition which one encounters among rabbits in rare instances. It has been designated by us "incompetence," as indicating an inability to develop an effectual reaction. The defective resistance of such animals is not entirely specific but is apparent in the reaction to injuries of many kinds which suggests a constitutional inferiority as an underlying cause. This assumption is of importance in the present instance as affording one basis for a possible explanation of the peculiar conditions presented by the offspring of the animal.

Female Parent.—Albino; weight at time of inoculation 575 gm. Inoculated Jan. 14, 1919 with the Nichols strain of *T. pallidum.*

This animal was one of a group of young rabbits (3 to 4 months old) inoculated by the intravenous injection of 1.0 cc. of a testicular emulsion rich in spirochetes. She proved to be one of the most resistant animals of the group; her health was little if at all affected by the inoculation. The only lesion noted was a periostitis of the nasal bones which developed about 8 weeks after inoculation; this lesion was comparatively small and healed in about 1 month.

The animal developed normally and attained a weight of 2,000 gm. by Sept., 1919. She was kept under observation until June, 1920. During this time she was mated twice with the rabbit whose history is given above and later with another infected animal.

As has been stated, the first of these matings was without issue while the second resulted in the birth of the six rabbits with which this report is concerned. The third mating was on Feb. 26, 1920. The male used in this instance was also an animal that subsequently developed a severe generalized syphilis. At the time of the mating, he presented an unusually marked acute orchitis involving both testicles but was still fertile, as a litter of six young was born of this mating. Four of these rabbits died soon after birth; the two others developed normally.

There is nothing in the history of this rabbit that is unusual. The two things that may be significant are the high resistance of the animal as contrasted with the low resistance of the male parent and a suggestion of impaired reproductive activity which may or may not be referable to syphilis.

The Litter of Young.—The litter of six young, obtained by the mating of the two rabbits whose history is given above, was born December 26, 1919; there were two males and four females, all albinos. At birth, three of the young were much smaller than the others but no other abnormality was noted until toward the end of the 2nd week of life.

When the eyes opened, it was found that one of the smallest animals of the lot, a female, showed an opacity of the entire cornea of the right eye (Fig. 1). The condition present was distinctly different from that seen in adult rabbits following inoculation with *Treponema pallidum* in that there was no pannus and only a suggestion of a pericorneal injection with no other evidence of an inflammatory reaction in any part of the eye. The cornea was grayish white with a slightly roughened surface; the deeper structures could not be seen.

A little later, a difference in the appearance of the coats of the six animals was noted. At the age of 3 weeks, this difference was very striking. Three of the rabbits, including the one with the corneal opacity, showed coats that were smooth and glossy (Fig. 2) while the coats of the others presented a roughened and unkempt appearance, due to an abnormal growth of hair with a tendency to mat together and to extensive shedding (Figs. 3 to 6). The parts chiefly affected were the trunk, the neck, and the proximal segments of the tail and legs. As the condition progressed, extensive areas of the body were completely denuded of hair so that by the end of the 4th and 5th weeks after birth there was a very striking contrast between those animals of the litter that were the subjects of this peculiar affection and those whose coats remained smooth and glossy. This is shown by a comparison of Figs. 3 to 6 with Fig. 2 which represents the animal with the corneal lesion.

The skin of these animals showed no evidence of an inflammatory reaction or of scratches or abrasions; on the contrary, it was smooth and delicate. All of the animals were well nourished and apparently in good health; they were free from vermin and there was no evidence of an alimentary disturbance of any kind. Moreover, the mother appeared to be in good health and her coat was in good condition for a lactating female. In brief, there appeared to be nothing connected with the diet or mode of living that could account for the condition presented.

This peculiar form of alopecia, if we may term it such, was of relatively short duration. By the end of the 6th week a new growth of hair appeared over the bare areas and the coats of all animals soon presented a normal appearance.

During February, one animal died as a result of a gastrointestinal disturbance. In May, all of the animals developed a dermatitis associated with alopecia for which no immediate cause could be found. The skin over the legs, about the face, and over parts of the ventral surface of the body became red and thickened; the surface of the skin was covered with fine silvery scales and yellowish brown crusts and the hair over these areas became extremely thin. It will be noted that the parts affected in this instance were those that were least affected by the earlier alopecia. A dermatitis of this kind is not un-

common among young rabbits that are poorly nourished or living in unsanitary surroundings and no particular significance could be attached to the occurrence of such a condition under these circumstances, but in the present instance the occurrence of dermatitis may be significant.

The subsequent development of four of the five rabbits was uneventful. The history of the fifth animal, the female with the corneal lesion, contains several points that are of interest. In the first place, the lesion of the cornea gradually cleared from the periphery until there was only a slight clouding in the region immediately overlying the pupil. It was then apparent that the pupil was fixed, small, and irregular, and that there were delicate adhesions between the cornea and the iris; the deeper structures showed no perceptible abnormality. Eventually, there was some relaxation or absorption of the adhesions so that the pupil acquired some power of accommodation. The opacity in the cornea also diminished until it was barely perceptible but never completely disappeared.

In addition to the eye defect, this animal presented a number of developmental peculiarities and pathological conditions that are of interest in the present connection. Among these, we may mention the following conditions: Retarded growth, subnormal development of the skeletal system, the accumulation of abnormal deposits of fat, delayed sexual maturity and subnormal development of reproductive functions including ability to rear young, abnormal growth tendencies in the tissues of the generative organs and mammary glands expressed in the formation of polypoid growths and multiple cysts with atypical epithelial proliferation, and finally, the occurrence of an umbilical and a diaphragmatic hernia. The extent of the cystic development in the mammary glands is shown in Fig. 7 while Fig. 8 shows the hernia at the umbilicus and the protrusion of a mass of fat into the right pleural cavity as well as the abnormal accumulation of fat elsewhere. In this connection it may be noted that the animal had been losing weight for several months and that at the time of autopsy her weight was 500 gm. below normal, a loss of approximately 25 per cent.

This animal was kept under close observation for 5 years during which time she was used in a number of breeding experiments. Even-

tually, she developed a respiratory infection which pursued a course identical with that of the male parent. She was killed for postmortem examination in January, 1925.

All that need be said at this time concerning the unusual combination of conditions presented by this animal is that such conditions are strongly suggestive of some profound constitutional defect which influenced both intrauterine and postnatal development.

Tests for Syphilitic Infection.

At the time our interest was first aroused in determining the presence or absence of syphilitic infection in the litter of rabbits whose history is given above, no reliable method had been developed for making such a determination. The only method then in use was that of a test inoculation to determine susceptibility to infection. This method is open to serious objection. Nevertheless, three of the five rabbits were tested in this manner on November 19, 1920, by the intracutaneous inoculation of the vulva with a spirochete emulsion.

All of the rabbits developed primary lesions comparable to those of control animals but in two of them the course of disease was more prolonged and more severe than usual; in spite of an intervening pregnancy and lactation, one of these animals developed marked generalized lesions while the chancre of the other remained active for more than 5 months.

While these tests do not disprove the existence of a previous infection, they do indicate that the animals were probably free from infection at the time of inoculation.

The female with the corneal lesion and the surviving male of the litter were not inoculated. They were held for observation and for breeding purposes until the spring of 1924, when tests for infection were made by the removal of a popliteal lymph node and the inoculation of emulsions of these nodes into the testicles of normal rabbits.

Negative results were obtained in both instances. This method is the most reliable one that has been developed for the determination of latent syphilitic infection in rabbits but negative results from a single passage are not absolutely conclusive. The most that can be said in such cases is that either no infection exists or that any organisms

present are too few or too attenuated to cause the development of recognizable lesions when introduced into the testicles of normal animals.

DISCUSSION AND CONCLUSIONS.

The conditions reported are unique in experimental syphilis and their interpretation is by no means certain. It is possible that the alopecia, keratitis, iritis and other abnormalities presented by these rabbits had no connection with a syphilitic infection—either in the parents or in the animals themselves—and that their occurrence was referable to some very unusual combination of circumstances of which we have no knowledge. On the other hand, there may have been an intrauterine infection which cleared up before or shortly after birth. Still another possibility that must be considered is the occurrence of some primary disturbance of nutrition in the mother which affected intrauterine development to some extent but was most marked in its effects during the first few weeks of life. Such a disturbance of nutrition may or may not have been influenced by syphilitic infection. Finally, one cannot lose sight of the fact that the sire of this litter of rabbits presented unmistakable evidence of a constitutional inferiority and that inheritance of these qualities may afford a basis for an explanation of the conditions noted in the offspring.

It is impossible to say which of these conceptions is correct as none of them is susceptible of experimental proof. The lesion of the cornea and iris is strongly suggestive of infection while the other conditions are more suggestive of nutritional or developmental abnormalities, but in either case syphilis may have been a factor of decisive importance.

At all events, the congenital lesion of the eye on the one hand and the peculiar growth of hair followed by an extensive alopecia on the other bear a resemblance to keratitis of congenital syphilis (which may occur as a congenital lesion), the so called syphilitic mop of Hutchinson and alopecia universalis of syphilitic origin, or to cases of syphilitic alopecia described by Wile (2) which are not due to a skin infection. The retarded and subnormal development of the animal with the eye defect is also suggestive of syphilis, but, from our knowledge of syphilis in the rabbit, it does not appear likely that any of these animals was actually infected.

If we eliminate infection of the young as the immediate cause of the conditions described, we must regard them as of functional origin due to some disturbance or defect in the mechanism which controls processes of growth and development. The problem would thus be narrowed down to the basis of parental influence with the alternative of attributing the conditions to a disturbance of nutrition in the mother or to inheritance of paternal defects or to a combination of the two sets of factors.

As has been said, the evidence available is not conclusive but the history and the tests of susceptibility to syphilitic infection point strongly to paternal inheritance as the factor of foremost importance. This view is also supported by further studies of heredity as a factor in determining resistance or susceptibility to syphilitic infection, based on a series of brother-sister matings which began with the male and two of the females described in this report.

If it be granted that inheritance of paternal characters may affect functional activities in the offspring, and this appears to be true in the case of the susceptibility of rabbits to syphilitic infection, the conditions described above may be accounted for satisfactorily as expressions of disturbed function. It has been found that syphilis in the rabbit is accompanied by marked alterations in many of the glands of internal secretion and that interference with the functional activity of these glands by surgical removal produces a profound effect on the course of the disease (3). Finally, it is generally believed that some of these organs play an important part in the regulation of growth and development. This applies to the teeth, the skin and its appendages, and the organs of special sense, as well as to other parts of the body and, as is well known, these structures are frequently affected by disturbances of function in the glands of internal secretion. Hence, it appears not unlikely that the conditions presented by this group of rabbits may be referable to abnormal functional activity of this system of organs, determined largely by inheritance but influenced also by the presence of a syphilitic infection in one or both parents.

In brief, therefore, it would appear that while under ordinary circumstances the resistance of the rabbit, and of females in particular, is of such a high order as to prevent the occurrence of intrauterine infection of the offspring, it is still possible for the normal course of pregnancy to be interfered with, and, in exceptional instances, the

young born of syphilitic parents may exhibit abnormalities suggestive of syphilis even though no infection can be demonstrated. The occurrence of such stigmata may be due to abnormal functional activity of a growth-regulating mechanism the character of which is determined primarily by heredity; but, the action of the mechanism may be affected directly or indirectly by the presence of a syphilitic infection.

SUMMARY.

The report is concerned with the occurrence of stigmata of syphilis in the progeny of syphilitic rabbits and the probable cause for such conditions in cases where no infection can be demonstrated.

The history of a group of animals presenting these conditions is given and possible causes are discussed in the light of evidence derived from a study of these animals.

In this instance, it appeared that the lesions suggesting syphilis were of functional rather than of infectious origin and that heredity was an important factor in determining their occurrence.

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1. Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndhtsamte.*, 1913, xliv, 307.
2. Wile, U. J., personal communication. Report to appear in *Arch. Dermatol. and Syphilol.*, 1925.
3. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 476.

EXPLANATION OF PLATES.

PLATE 27.

FIG. 1. Right eye, showing corneal lesion of the animal in Fig. 2.

FIG. 2. Normal coat. 4th week after birth.

FIGS. 3 and 4. An animal of the same litter as the one in Fig. 2. Note the abnormal growth of hair, the tendency to mat, and the extensive loss of hair. 4th and 5th weeks respectively.

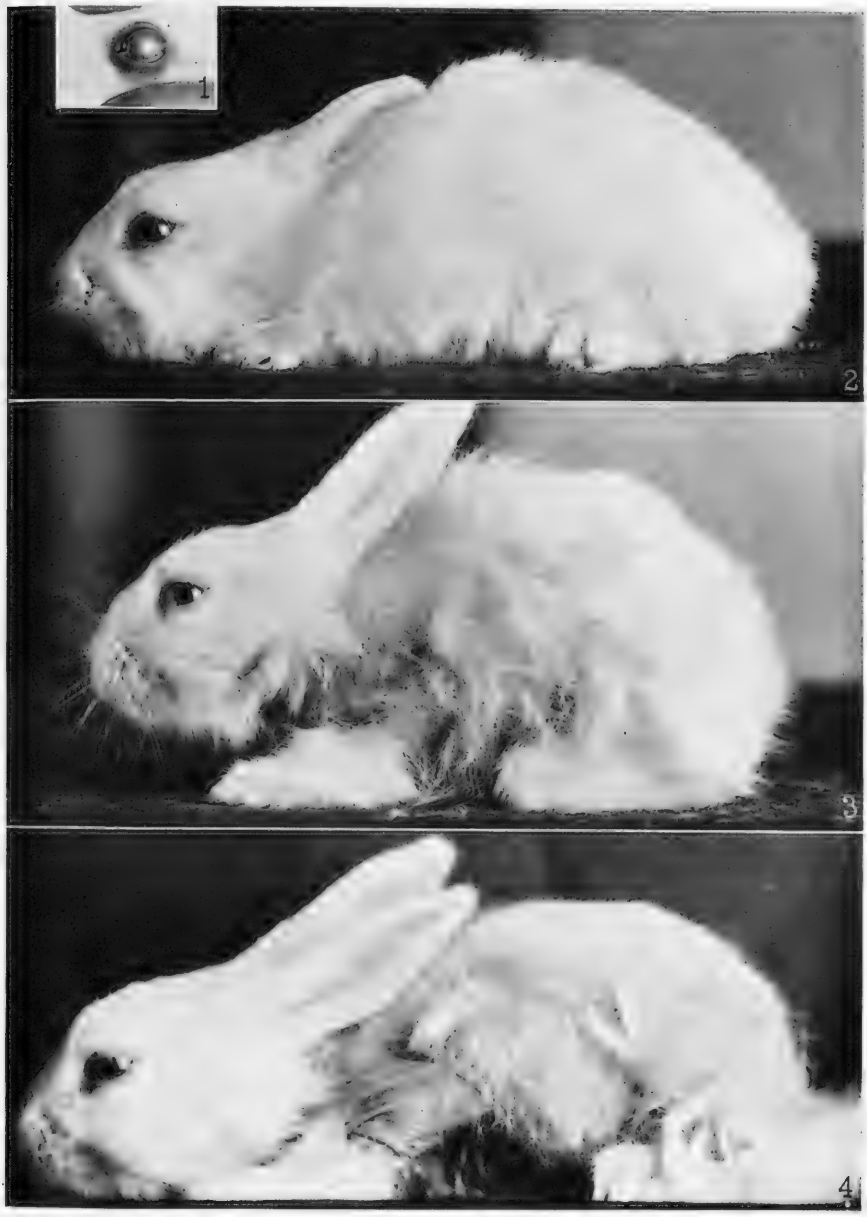
PLATE 28.

FIGS. 5 and 6. Another animal of the same litter at corresponding times.

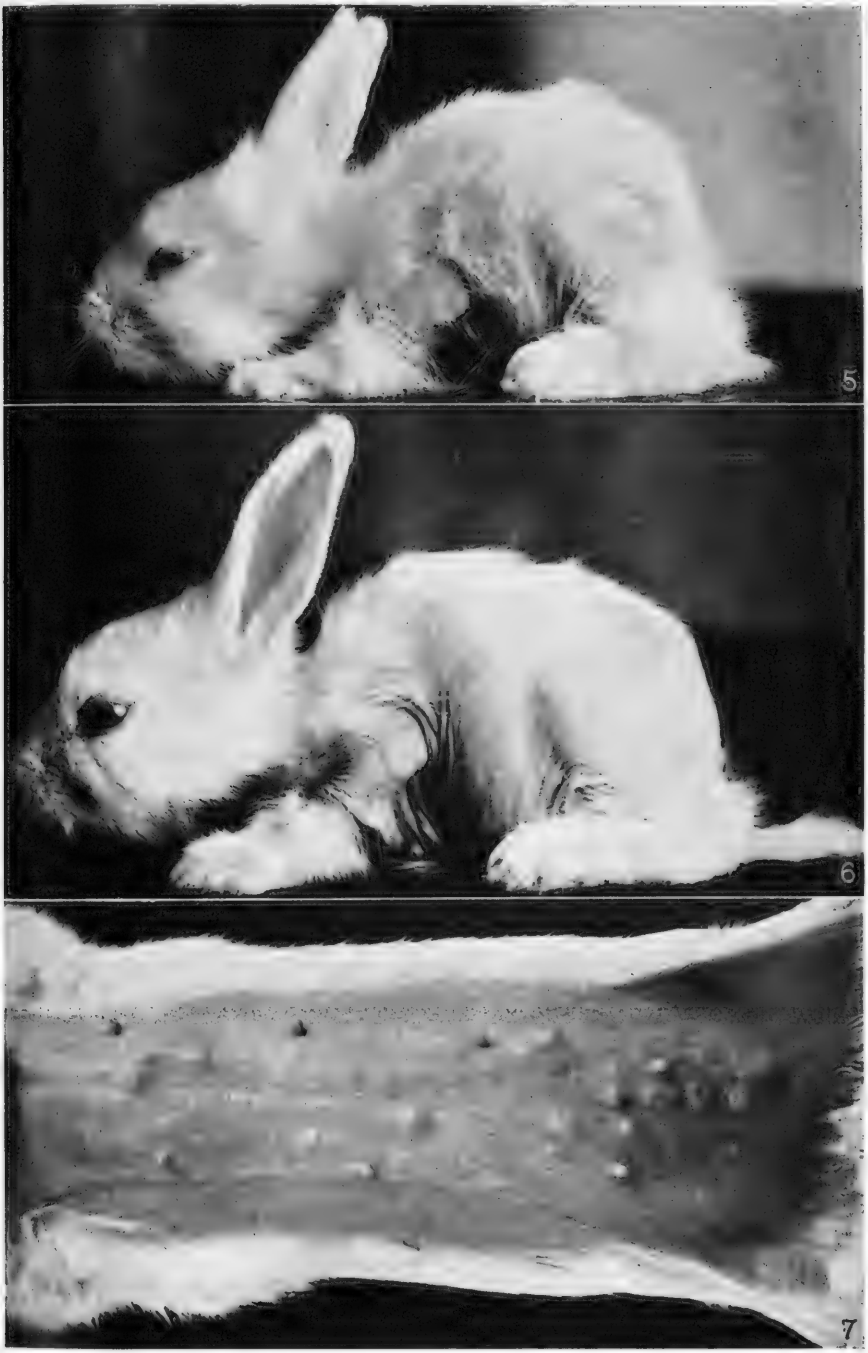
FIG. 7. Multiple cysts of the mammary glands. The same animal as that in Figs. 1 and 2 at the age of 3½ years.

PLATE 29.

FIG. 8. The same animal as in Fig. 7. 1½ years later. Note the umbilical hernia, the protrusion of a mass of fat into the right pleural cavity, the presence of lobulated and pedunculated masses of fat in the subcutaneous depots, and the excessive accumulation of fat in an animal showing a loss of weight amounting to 25 per cent.



(Brown and Pearce: Stigmata of congenital syphilis.)



(Brown and Pearce: Stigmata of congenital syphilis.)

(Brown and Pearce: Stigmata of congenital syphilis.)

EXPERIMENTAL PRODUCTION AND TRANSMISSION OF TAR SARCOMAS IN CHICKENS.

By JAMES B. MURPHY, M.D., and KARL LANDSTEINER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 30 to 32.

(Received for publication, April 6, 1925.)

The experiments described in the following pages were undertaken with the view of gaining information as to the filterable causative agents of transplantable chicken tumors. This group of neoplasms,[1] representing a variety of typical sarcomas, resembles in all particulars, histologically and biologically, the transplantable tumors of other animals, yet many, if not all, of those closely studied have the unique quality of being transmissible through the agency of a cell-free filtrate[2],[3] or a desiccate of the tumor. Furthermore, the distinctive histological and biological characters of the individual neoplasms are transmitted equally as well by the filtrate or desiccate as by the living tumor cells. The nature of these filterable agents has been discussed repeatedly because of their fundamental significance in the general question of tumor etiology. Four main conceptions have been put forward to explain the facts but two of them, namely that the chicken sarcomas are inflammatory conditions resulting from infection, not true tumors, and the other that the viable neoplastic cells pass through the filters or resist drying, are so unlikely that they need not be discussed. Rous has been inclined to interpret the evidence as indicating that the causative agents are in the nature of filter-passing organisms. Another interpretation hinted at by some authors

[1] Fujinami and Inamoto, *Verhandl. jap. path. Ges.*, 1911, l, 114. Rous, P., *J. Exp. Med.*, 1910, xii, 696. Tytler, W. H., *J. Exp. Med.*, 1913, xvii, 466. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651.

[2] Rous, P., *J. Exp. Med.*, 1911, xiii, 397. Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lix, 1793.

[3] Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52.

(*i.e.*, Doerr[4]) presumes the existence in these tumors of a substance produced by the tumor cells and causing the malignant transformation of other cells in a manner analogous to the action of the d'Hérelle substance which produces changes in the bacteria with which it is associated, appears to multiply in such association, and is transmitted to subsequent generations.

Our purpose was to engender in chickens a malignant tumor, preferably a sarcoma, by artificial means and to investigate whether or not such a tumor would contain a filterable agent capable of transmitting the tumor. A positive finding might be taken to favor the conception that an inanimate principle is responsible for tumors. Whatever the outcome, however, it would have an interesting bearing on the subject.

The method selected was the injection into fowls of an extract from the residue of distilled coal tar. It is well known that such a substance applied repeatedly to the skin will induce carcinoma,[5] but recently Russell has shown that the subcutaneous injection of it in rats is quite as regularly followed by the development of sarcoma.[6] There seems to be no doubt that the experimentally induced tumors are true neoplasms. Histologically they have the same characteristics as the spontaneously developed growths. They metastasize more or less freely, and some of them are transplantable though as a group they are subject to the same limitations in this respect as spontaneous tumors.

Production of Tar Tumors in Chickens.

As it was desirable for our purposes to secure a sarcoma rather than a carcinoma, the tar preparation was injected instead of painted on the surface. With the hope of hastening the process hashed up tissues of chicken embryos were added to the first tar injected and the later injections were made into the embryomas that developed. The tar product used had been previously found to be extremely active in the production of cancer in mice.

[4] Doerr, R., *Klin. Woch.*, 1922, i, 1489, 1537.

[5] Yamagiwa and Itchikawa, K., *Gann. Japan Z. Krebsforsch.*, 1916, x, 1. Tsutsui, H., *Gann. Japan Z. Krebsforsch.*, 1918–19, xii, 17. Fibiger, J., and Bang, F., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1157.

[6] Russell, B. R. G., *J. Path. and Bact.*, 1922, xxv, 409.

Preparation of Tar.—The tar product used throughout these experiments was prepared from a residue obtained from a coke oven in which the tar had been distilled at a temperature of approximately 377°C.[7] About 250 gm. of the hard, black, pitch-like residue was pulverized to a fine powder and extracted with 2½ liters of benzene for 24 hours. It was then filtered to remove the undissolved particles and the filtrate distilled until the volume was reduced to approximately 500 cc. The tarry residue was then washed with 1 liter of a 12 per cent NaOH solution in water and the same procedure was repeated with 1 liter of 30 per cent H_2SO_4. The heavy precipitate thrown down by these treatments was removed by gently warming the mixture on a steam bath until the precipitate coalesced, when the liquid fraction was decanted. The latter portion was washed four times with water. The tar fraction which remained in the benzene solution was evaporated to drive off the excess benzene, leaving a residue of about 300 cc. of the product.

Experiment 1.—Ten full grown Rhode Island Red hens were injected in the right breast with 0.3 cc. of hashed 7 day old chick embryos and in the left breast with 0.5 cc. of a mixture of equal parts of tar residue and hashed embryo. At 2 week intervals thereafter, throughout the duration of the experiment, 0.2 cc. of the tar was injected into the resulting mass in the left breast. The rate of growth of the embryonic tissue in the two sides showed considerable variation. In four birds the larger nodule developed in the tar-injected side, while in four the embryonic tissue alone grew more rapidly, and in two the growth was about equal. With one exception the normal embryomas reached their maximum size in from 1 to 2 months and then slowly retrogressed to complete absorption. One of these, however, continued to grow for 5 months, by which time it had attained a size of 5.6 by 4.3 cm., when it became stationary and has since shown no growth. Pieces removed at operation have shown it to be made up of bone and cartilage.

By the end of 5 months only three fowls remained alive and two of these had progressively enlarging tumors in the tar-injected side.

Chicken Tumor A.—In Chicken 1 the growth started after a period of retrogression of the embryoma. During the 5th month the nodular mass characteristic of the embryoma was replaced by a smooth well rounded tumor which continued to grow progressively till it caused the death of the animal. Small pieces were removed from time to time for histological examination. Hereafter we will refer to this neoplasm as Chicken Tumor A.

At autopsy the left breast muscle was almost completely replaced by a large tumor composed of two rounded masses connected by a wide base. The larger measured 5 to 6 cm. in diameter and the other about 4 cm. In places the tumor was bordered by reactive tissue for the most part associated with the remains of the tar. The tumor

[7] We are indebted to the Barrett Company for the preparation of this product.

was firm, almost gristly, sharply differentiated from the muscle, homogeneous, semitranslucent, with a slightly yellow tinge. There was no necrosis or hemorrhage. Tar droplets were found scattered through it. The remains of the old embryoma persisted in the right breast as a thin yellowish placque about 5 by $3\frac{1}{2}$ cm. and less than $\frac{1}{2}$ cm. thick. There were deposits of fat scattered through this firm mass which on section appeared to be made up of bone and cartilage. No gross metastases were found in any of the organs. Some congestion of the lungs was present and the liver was mottled with whitish areas from pin-point to pin-head size. The egg duct contained a normal sized egg with the shell crushed but the membrane intact. The other organs appeared normal.

Histologically the tumor was made up of medium size spindle cells, closely compacted and coursing in irregular bundles. The remains of the embryoma, composed mostly of bone and cartilage, were found at the edge of the tumor. No microscopic metastases were noted.

Chicken Tumor B.—Chicken 9 showed the beginning of an active tumor growth in the tar-injected side by the end of the 3rd month and this progressed till it caused the death of the fowl by the 7th month of the experiment. This neoplasm will be designated Chicken Tumor B.

At autopsy a large tumor was found in the left breast, measuring 8.1 by 6.5 cm., almost replacing the pectoral muscles (Fig. 1). In the skin of the right breast there was a nodule about the size of a pea, the result of an autotransplant introduced by us several weeks previously. A crater-like cavity in the middle of the primary tumor was filled with blood and necrotic debris. On cutting through the tumor it was found to be uniformly grayish white in color, firm to the touch but easily crushed. There was no necrosis except a small area associated with the cavity resulting from the removal of a piece of the tumor. The borders were sharply defined from the muscle but the striæ occurring in the tumor followed the same general line as the fibers of the muscle. The liver contained a rounded mass approximately 2 cm. in diameter and two smaller ones (Fig. 2). On section the larger showed a beginning necrosis in the center with radiating lines extending to the periphery. The lungs were almost replaced by firm whitish nodules varying from the size of a pin-head to that of a large pea (Fig. 3). In general consistency the hepatic nodules were similar to the nodules in the lungs. The other organs appeared normal.

Histologically the tumor tissue was of rather uniform appearance. It consisted of irregular, interlacing bundles of slender spindle cells, and a small amount of intracellular slightly undulated fibrils. The tumor cells did not vary much in shape or size but a small number of rounded forms were noted, sometimes containing more than one nucleus. The typical nuclei were long and thin, some rod-like. Mitosis was rare. There were a few foci of mononuclear round cells and some lacunæ, from which doubtless tar remains had dissolved out, surrounded by foreign body giant cells. In places on the surface the connective tissue was honeycombed with such lacunæ, enclosed by the same type of giant cells. The cells and the general structure of the metastases resembled the original tumor but more rounded elements were present in the lung metastases, and the spindle cells were in general shorter and thicker.

Transplantation Experiments. Chicken Tumor A.

At intervals during the development of Chicken Tumor A pieces were removed and transplanted into other Rhode Island Red fowls from the same stock. In all, 56 chickens were inoculated, 26 of these being young fowls from 5 weeks to 3 months old. Not a single progressively growing tumor resulted, although temporary growth with later retrogressions took place in a few cases.

Transplantation of Chicken Tumor B.

The same general procedure was carried out with this tumor.

First Generation.—In all, 50 adult Rhode Island Red chickens received grafts from Chicken Tumor B and in several of these a temporary nodule developed but with one exception they retrogressed. In this one chicken, however, the tumor grew continuously but with great slowness so that at the end of 3 months it was only about 1.5 cm. in diameter, but during the next month active growth began and proceeded with great rapidity till the whole breast was replaced. Autopsy showed a tumor similar in general character to the original one, both in the gross and microscopically, but no metastasis had taken place.

Second Generation.—Grafts from the single tumor arising in the

first generation were grafted into both breasts of 15 young and 19 adult Rhode Island Red chickens. Of these 17 developed tumors which grew progressively in 14 instances while in 3 they retrogressed after attaining considerable size. Several of the tumor-bearing chickens were killed early but of those allowed to survive 7 showed metastases. The lung was involved in 6 cases, the heart in 3, the ovary in 3, the peritoneal cavity in 2, and the liver and stomach in 1.

Third Generation.—Transplants from the preceding generation yielded tumors in 8 out of 13 chickens inoculated and in all except 1 of these they grew progressively. The lungs were involved in 4 instances and the heart, liver, stomach, skin, muscle, and peritoneal cavity in 1 instance each.

The tumor has been carried in this manner through eleven generations, during which passages there has been some increase both in the growth rate and in the number of takes. The general character of the growth has not changed except that in the more rapidly growing tumors there is a greater amount of necrosis, of a dry yellowish character. We have encountered no cystic degenerations; and hemorrhages into the tumor are rare and never extensive. It is not uncommon to find that tumors growing in the breast muscle tend to follow the large vessels up towards the axilla and in several instances the tumor has extended through the axilla to the pericardium (Fig. 4). Occasionally we have observed scattered nodules on the outer surface of the esophagus extending down to and involving the pouch about the stomach. The distribution suggests that the tumor had been disseminated through the lymphatics.

Of the 77 fowls having progressive tumor something over 50 per cent had metastases. The lungs were involved in 31, the ovaries in 11, the heart in 7, the stomach in 7, the intestines in 6, the pericardium in 4, and the liver in 6. In 1 instance each, the skin, trachea, striated muscle, crop, and pancreas, was the site of metastasis. With the exception of the original chicken no discrete metastases have been found in the liver, the involvement in all of the 6 instances being due to direct extension of the tumor through the chest wall or the diaphragm.

Histologically the structure of the tumor in the later generations is similar to that of the original growth except for a greater irregularity

in the form and size of the cells (Fig. 5). Many of these are plump and irregular in outline, the nucleus large and intensely stained, and some are pycnotic. By incomplete division, giant cells with several or many nuclei are formed. Variability in size and shape of the cells is more evident in the metastases than in the primary tumor. Mitosis is frequent, in some tumors remarkably so (Fig. 6). Necrotic areas occur frequently and in some instances only a thin layer of tumor cells is found on the surface of the tumor mass.

The invasive character of the growth is evidenced by the infiltration and destruction of various tissues as muscle, fat, liver, constituents of the normal structure being surrounded and widely separated by the malignant growth. This is most strikingly seen where striated muscle is being replaced by the sarcomatous tissue, for here the tumor cells not only grow in the spaces between the fibers but invade the fibers themselves (Fig. 7). Frequently only remnants of the muscle fibers are to be seen either in the center or on the circumference of the sarcomatous cast. In such places, the structure simulates in cross-section a sarcoma with polygonal cells and alveolar structure, but on longitudinal section the spindle shape of the plump cells is still recognizable.

Filtration and Desiccation Experiments.

The tumor described here is not so active as one of the three types of sarcomas,—spindle-celled sarcoma, osteochondrosarcoma, and intracanalicular sarcoma,—studied at The Rockefeller Institute and perhaps metastasizes with less regularity; yet it is far more malignant than two other types of tumor described. In general behavior and histological appearance the tar tumor is most like the simple spindle-celled sarcoma of the group just mentioned, but a notable difference is to be observed in the fresh specimen as compared with this transplantable tumor of spontaneous origin. The latter tumor is usually soft and contains great quantities of mucoid material, while the tar tumor is much firmer and is free from any mucilaginous material. The general character, however, of the tar tumor unquestionably justifies grouping it with the other transplantable chicken sarcomas and all of these closely studied have proved to be transmissible through the agency of a cell-free filtrate or desiccate of the tumor material.

A priori there would seem to be every reason to expect that the tar tumor would be found to possess the same quality.

Filtration Experiments.—About 20 gm. of the tumor material relatively free from necrosis was chopped into fine bits and thoroughly ground with sterile sand. About 400 cc. of Ringer's solution was added and the suspension shaken for 20 minutes. It was then centrifuged to remove the sand and solid particles, and the supernatant fluid decanted. A small amount of a suspension of *Bacillus prodigiosus* was added immediately before filtration in order to test the filters. The extract was then passed, part through a Berkefeld N and part through a Berkefeld V filter. Sterile infusorial earth (Kieselguhr) was added to each of the filtrates and from 5 to 10 cc. injected into the breast muscles of normal Rhode Island Red chickens. Cultures of the filtrate on agar remained sterile, showing that the filters were impervious to *Bacillus prodigiosus.*

This filtration test was carried out seven times with material from seven different tumors selected from the first to the eleventh generations and, in all, 50 chickens have been inoculated with filtrate. The fowls were followed for from 3 to 5 months, and in a few instances still longer. Not one tumor developed.

Desiccation Experiments.—A quantity of fresh tumor material was chopped fine, spread in a thin layer on a glass plate, and placed in a desiccation jar over sulfuric acid. The air was evacuated and the jar placed in a freezing room till the tumor was completely dry (24 to 48 hours). The material was then ground to a fine powder, taken up in Ringer's solution, and injected into the breast muscles of normal chickens.

This test has been carried out with five different tumors and 42 chickens have been injected with the material. None of these have developed tumors during the period of observation (3 to 6 months).

DISCUSSION.

The immediate object of the experiments has been accomplished in that a sarcoma of the chicken has been engendered by experimental processes which in its histology and its general behavior on transplantation resembles closely some of the spontaneous chicken tumors. The tar tumor fulfills all the criteria necessary to identify it as a neo-

plasm. It grows with considerable rapidity, metastasizes freely, and is highly invasive in character. It is as typical a neoplasm as the transplanted chicken tumors of "spontaneous" origin. Nevertheless we have been unable to demonstrate in association with the growth a tumor-producing substance separable from the tumor cells. It can be transplanted at the present only by the employment of living cells. However, the data thus far obtained have the limited value of negative evidence, for the possibility cannot be excluded that another tar tumor or a different technical procedure might yield a positive result.

A point of perhaps fundamental importance should be mentioned; namely, the suitability of the soil necessary for the effective action of tumor filtrates. The causative agent of the chicken tumor which this growth so much resembles (Chicken Tumor I) is capable of producing malignant transformation in the mesodermal tissue of the embryo,[8] having no effect on the ectodermal or endodermal tissues, and in the adult [3,9] it gives rise to tumors only when brought in contact with young or reactive connective tissue. The tar tumor considered in the present paper had its origin in embryonic tissue and quite possibly filtrates from it may be capable of affecting only a specific type of embryonic cell. This problem is under investigation and attempts are also being made to obtain other strains of tar tumors.

SUMMARY.

Two spindle cell sarcomas have developed as the result of the injection of coal tar into induced embryomas in chickens. One of these, transplanted to other chickens for eleven generations, is highly invasive in character, and metastasizes freely. Attempts to transmit this neoplasm by a filtrate or desiccate of the tumor have failed of success.

EXPLANATION OF PLATES.

PLATE 30.

FIG. 1. Lateral view of the chest wall of Chicken 9 (Chicken Tumor B) showing the original tumor induced by the injection of tar. The tumor has been sliced

[8] Murphy, Jas. B., and Rous, P., *J. Exp. Med.*, 1912, xv, 119.

[9] Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lviii, 1751.

through showing (*A*) healthy tumor, (*B*) necrotic area, (*C*) cavity containing the blood clot resulting from an earlier operation.

FIG. 2. Liver from the same animal showing two small and one large metastases indicated by arrows.

PLATE 31.

FIG. 3. Lung from the same animal. The white areas are metastases which almost replace the entire lung.

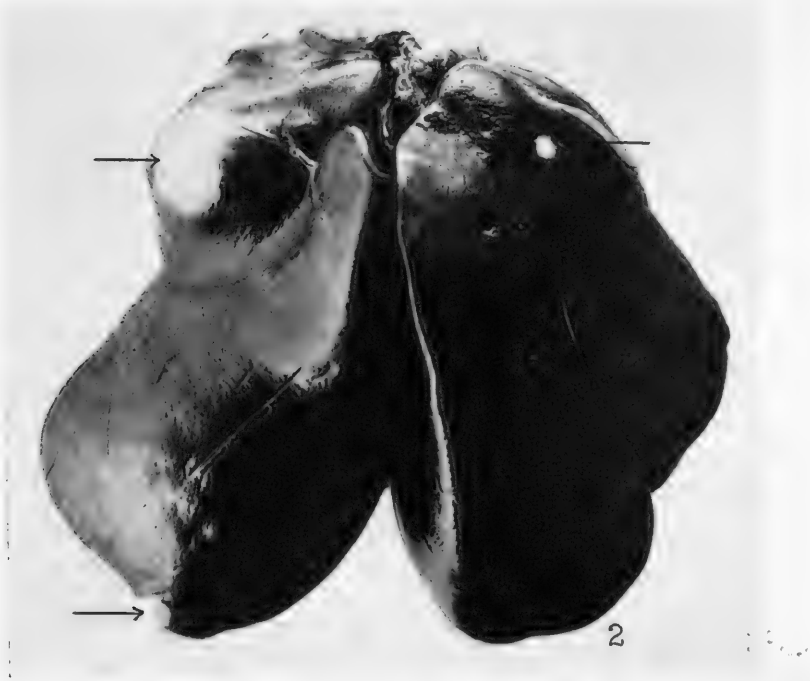
FIG. 4. Heart from a chicken inoculated in the breast muscle with a transplant from the tar-induced tumor (third generation). The tumor in this case followed the large vessels through the axilla and down to the heart. *A*, mass of tumor surrounding the large vessels; *B*, metastasis in the heart muscle.

FIG. 5. A photomicrograph of a section taken from a later generation of the tar tumor.

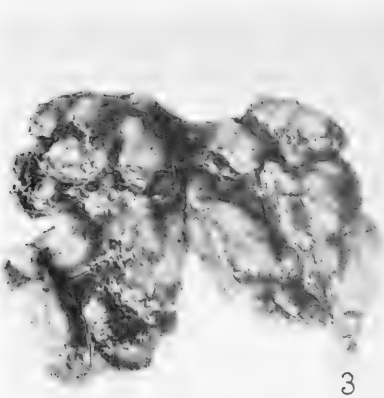
PLATE 32.

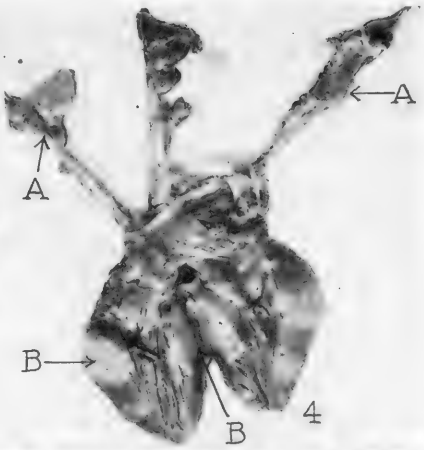
FIG. 6. A section of a transplant of the tar tumor showing a remarkable number of mitotic figures.

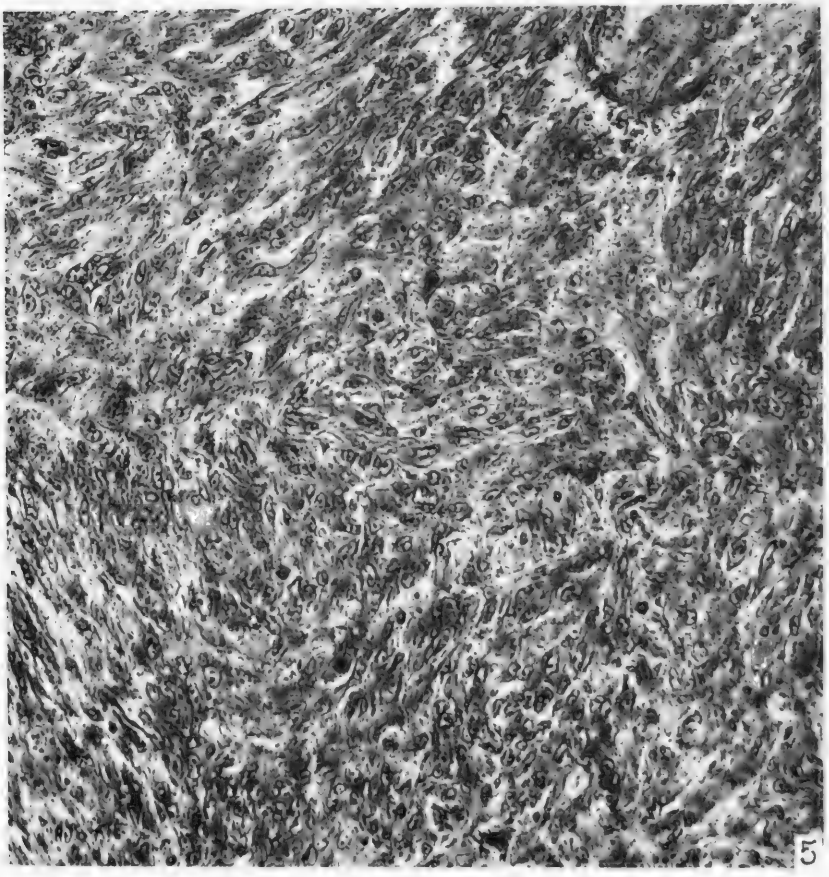
FIG. 7. Section showing the tar sarcoma invading striated muscle. *A*, remains of muscle fibers partly invaded by tumor cells; *B*, muscle fibers completely replaced by tumor cells.



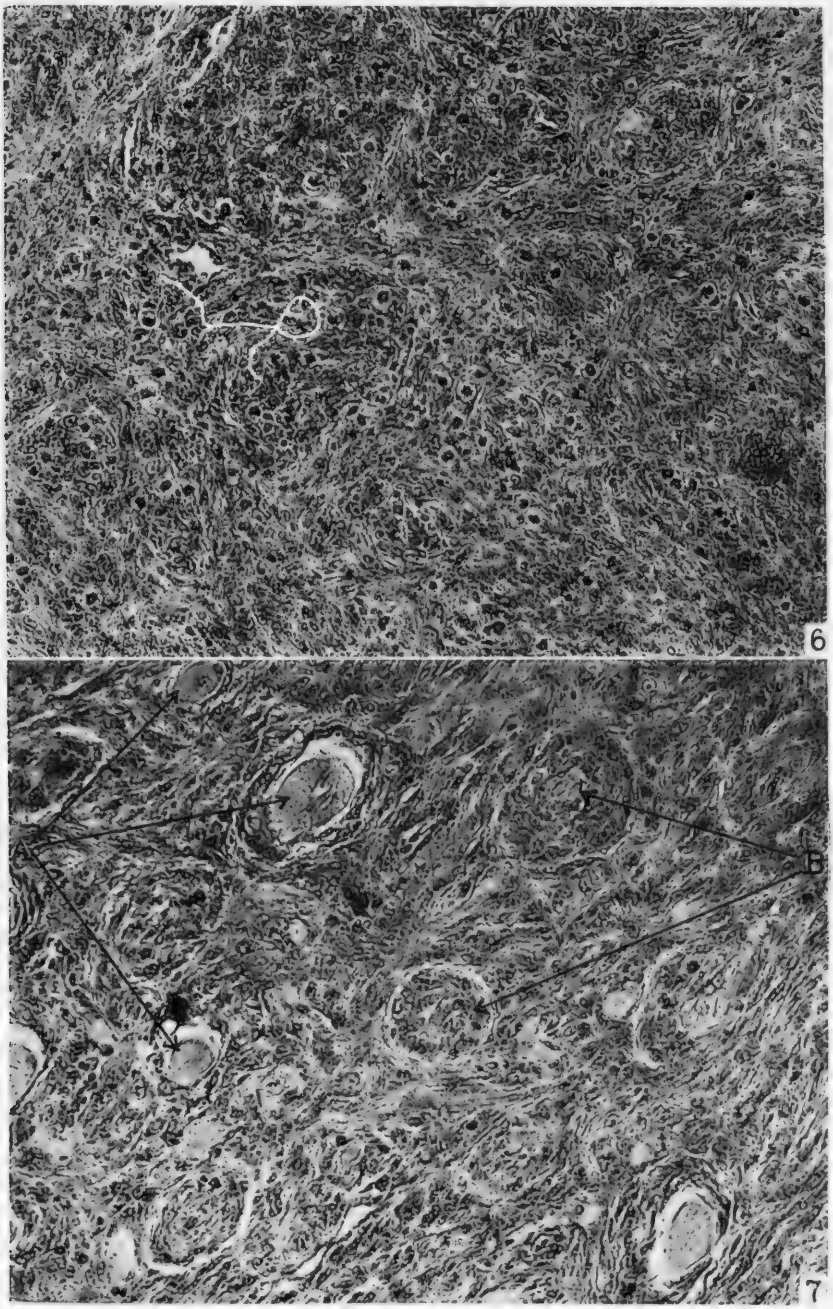
(Murphy and Landsteiner: Tar sarcomas.)







(Murphy and Landsteiner: Tar sarcomas.)



(Murphy and Landsteiner: Tar sarcomas.)

A GROUP OF MICROORGANISMS TRANSMITTED HEREDITARILY IN TICKS AND APPARENTLY UNASSOCIATED WITH DISEASE.*

By E. V. COWDRY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 33 to 35.

(Received for publication, March 9, 1925.)

In the course of a study of the relation of *Rickettsia ruminantium*[1] to heartwater, it was found that a microorganism previously reported in *Amblyomma hebræum*[2] was present not only in the eggs of adult females, but also in larvæ hatched therefrom and in nymphæ. With the unusual opportunities afforded for the investigation of all stages in the life history of ticks in the laboratory of the Department of Agriculture of the Union of South Africa, an attempt was made to ascertain whether this microorganism in *Amblyomma hebræum* afforded an isolated example of hereditary transmission or was one of a group of parasites thus transmitted.

Attention was first directed toward the study of unfed larvæ because the probability seemed to be a strong one that any microorganisms found in them must have been inherited through the eggs, since the larvæ had hatched out from eggs deposited by the female in sterile test-tubes plugged with cotton which were carefully kept from contamination.

Soon somewhat similar microorganisms characterized by relatively

* Third contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made in the Laboratory of the Department of Agriculture at Onderstepoort. Cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler, and to the members of his staff for the many courtesies extended.

[1] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii (in press).

[2] Cowdry, E. V., *J. Exp. Med.*, 1923, xxxvii, 431.

large size as compared with most *Rickettsiæ*, great pleomorphism, Gram-negative properties, and intracellular habitat were observed in both smears and sections of the unfed larvæ of *Boophilus decoloratus*, *Hæmophysalis leachi*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi*, and *Rhipicephalus simus*, in addition to *Amblyomma hebræum*. Fortunately these six species of ticks were being reared in the laboratory for other purposes so that material was at hand in which to trace the history of the microorganisms through some of the subsequent stages of development. Photographs of the microorganisms, as seen in smears, are given in Figs. 1 to 6; camera lucida drawings, illustrating their degree of pleomorphism, are reproduced in Figs. 7 to 12; and their appearance in sections is shown by the drawings in Figs. 13 to 18.

The survey was extended by the inclusion of certain phases in the life history of other ticks available in the laboratory and by a re-examination of specimens previously described.[1,2] In this way it was found that microorganisms of the same general type, and probably also transmitted hereditarily, were present in at least sixteen species. These are denoted by the plus (+) signs in Table I, which gives full information regarding the material examined. Unless there is a statement to the contrary, it is to be understood that the ticks were obtained in South Africa. To these the laboratory numbers are appended for reference. The methods employed in handling the larvæ, nymphæ, and adults, and the technique applied were the same as those used in an earlier study on *Rickettsia ruminantium* in ticks.[1]

The microorganisms could not be detected by direct illumination of living cells with or without the addition of vital dyes with sufficient facility and constancy to delimit their properties, although in favorable preparations they were clearly recognizable.

Dark-field examination[3] of living cells revealed only a few of the morphological forms in the case of each species of tick. In some cases the entire microorganisms appeared brightly luminous, while in others the luminous material was confined to a thin marginal layer. In no instance was definite motility observed.

In air-dried smears after fixation for about 15 minutes in absolute alcohol the microorganisms stained light red or pink with Giemsa's stain, that is to say colored much more faintly than most bacteria, and they did not possess the sharp

[3] With the precautions previously described.[1]

contours often seen in the case of bacteria. They were very pleomorphic, varying from spherules to straight and curved rods and filaments. They were found to be larger than most *Rickettsiæ* particularly in their diameter, which varied from about 0.3μ to 1.5μ. Filaments were frequently met with, sometimes as much as 5μ in length, or longer, in the case of *Boophilus decoloratus*, in which branching was noted (Fig. 2).

Group Characteristics of the Microorganisms in Unfed Larvæ.

Through the examination of living cells, of smears, and of sections it was found that the microorganisms in different species resembled each other in (1) their relatively large size as compared with most *Rickettsiæ;* (2) the restriction of their intracellular habitat to the Malpighian tubules, and to the eggs of the females; and (3) certain of their staining reactions.

Their ends were generally uniformly and evenly rounded; but occasionally they were sharply pointed. This slight affinity for stains and pleomorphism served as a basis of differentiation between the microorganisms and certain refractile coccoid, rod-like, and dumb-bell-shaped concretions contained in the Malpighian tubules which otherwise might have constituted a source of error.

After fixation in Regaud's fluid the microorganisms in sections were colored dark red or purple by Giemsa's stain. They were uniformly Gram-negative in both smears and sections. They stained feebly by Unna's alkaline methylene blue and other basic aniline dyes. They were colored faintly red by Pappenheim's pyronine-methyl green method. By all methods of staining, when they were embedded in cytoplasm, some were observed to exhibit distinct halos. It was also in sections that their intracellular habitat was most clearly distinguished. Their location was very characteristic, being restricted to the cytoplasm of the epithelial cells of the Malpighian tubules of larvæ, nymphæ, and adults. Occasionally they were seen in the cells lining the rectal sac, which is a continuation of the Malpighian tubules. They were found also in the eggs of adults of ten species. In no instance were they found to invade other tissues.

Differential Properties of the Microorganisms in the Different Species of Unfed Larvæ.

While possessing certain group characteristics in common, as indicated above, it was found possible to distinguish clearly the microorganisms present in each species of tick by characteristic differences in their morphology alone.

TABLE I.

Material Examined.

Species.	Unfed larvæ.	Nymphæ.	Adults.
Amblyomma americana +			Sections of 2 from Columbia, S. C., 5 from Washington, N. C., and 10 from Baton Rouge, La., fixed in Regaud's fluid.[2]
Amblyomma hebræum +	Smears of 25 and sections of 25 fixed in Regaud's fluid (517); dark-field examination; also 112 engorged larvæ prepared by a variety of methods.[1]	300 prepared by many methods, some infected with heartwater.[1]	Sections of 20 fixed in formalin and Regaud's fluid;[2] also sections of 6 infected with heartwater.[1]
Amblyomma maculatum −			Sections of 6 from Vancleave, Miss., fixed in formalin and Regaud's fluid.[2]
Amblyomma tuberculatum −			Sections of 1 from Perkinston, Miss., fixed in formalin and Regaud's fluid.[2]
Argas mineatus −			Sections of 2 from Southerland, Fla., fixed in Zenker's and Regaud's fluids.[2]
" *persicus* +	Smears of 25 and sections of 25 fixed in Regaud's fluid; also dark-field examination.		

Boophilus decoloratus +	Smears of 50, sections of 50, and dark-field examination of several, some infected with redwater (517, 570, 608, 622).		Sections of 10 fixed in formalin and Regaud's fluid.[2]
Dermacentor albipictus −			Sections of 2 from Regina, Canada, fixed in formalin and Regaud's fluid.[2]
Dermacentor variabilis +			Sections of 5 from Urbana, Ill., and 8 from College Park, Md., fixed in formalin and Regaud's fluid.[2]
Dermacentor venustus +			Sections of 9 from Hamilton, Mont., and 2 from Wawawai, Wash., fixed in Regaud's fluid and some infected with Rocky Mountain spotted fever.
Hæmophysalis leachi +	Smears of 25 and sections of 25 fixed in Regaud's fluid; also dark-field examination (629).		
Hæmophysalis leporis palustris −			Sections of 19 from Raleigh, N. C., fixed in formalin and Regaud's fluid.[2]
Hyalomma ægyptium +	Sections of 25 fixed in alcohol.		Sections of 5 fixed in Regaud's fluid; also dark-field examination.

TABLE I—*Concluded.*

Species.	Unfed larvæ.	Nymphæ.	Adults.
*Margaropus annulatus** +			Sections of 8 from Columbia, S. C., 10 from Washington, N. C., and 2 from Baton Rouge, La., fixed in Regaud's fluid.[2]
Margaropus annulatus australis +			Sections of 6 from Jamaica, fixed in formalin and in Regaud's fluid. Sections of 3 from Trinidad, fixed in the same way.[2]
Margaropus winthemi −	Sections of 25 fixed in alcohol.		
Ornithodorus megnini +	Sections of 25 fixed in Regaud's fluid; also dark-field examination.		
Ornithodorus moubata −	Sections of 25 fixed in alcohol.		
Ornithodorus turicata +			Sections of 1 from Arizona, fixed in Regaud's fluid.[2]
Rhipicephalus appendiculatus +	Smears of 25 and sections of 25 fixed in Regaud's fluid; also dark-field examination (621).		Smears of 1 and sections of 5 fixed in Regaud's fluid. Some infected (?) with anaplasmosis, others exhibited many bacteria in tracheal system (602, 612).

Rhipicephalus capensis −	Sections of 25 fixed in alcohol.		
Rhipicephalus evertsi +	Smears of 10, also dark-field examination; sections of 25 fixed in alcohol and of 25 fixed in Zenker's fluid (633).	Smears of 2 and sections of 11 fixed in Regaud's fluid and infected with East Coast fever (644, 645, 646, 650).	Sections of 20 fixed in formalin.[2]
Rhipicephalus pulchellus +		Sections of 2 fixed in Regaud's fluid and infected with East Coast fever (618).	Smears of 1, infected with East Coast fever (639).
Rhipicephalus sanguineus +			Sections of 14 from Hawaiian Islands fixed in formalin and Regaud's fluid;[2] also smears of 3 (555).
Rhipicephalus simus +	Smears of 25 (624), sections of 25 fixed in alcohol, and of 25 fixed in Regaud's fluid; also dark-field examination.	Smears of 2 and sections of 2 fixed in Regaud's fluid and infected with anaplasmosis (611).	Smears of 7 and sections of 6 fixed in Regaud's fluid and infected with anaplasmosis (561, 564).

* The generic term *Margaropus* is retained intentionally. It was supplied by Dr. F. C. Bishopp of the Bureau of Entomology of the U. S. Department of Agriculture and was employed by me in an earlier paper.[2]

Thus, in *Amblyomma hebræum* (Figs. 1, 7, and 15) the microorganisms were noticeably plumper and of larger diameter than in the case of any of the other ticks. They were, moreover, often grouped together in an unique fashion suggestive of multiplication by some kind of budding.

In *Boophilus decoloratus* the microorganisms differed from those in the other ticks by showing marked red- and blue-staining materials (Fig. 8), by the fact that some of the morphologic types were colored rather intensely by Giemsa's stain (Fig. 8), and by their branching (Fig. 2). They were never grouped together after the fashion of the microorganisms in *Amblyomma hebræum*, *Hæmophysalis leachi*, *Rhipicephalus appendiculatus*, and *Rhipicephalus evertsi*.

The microorganisms in *Hæmophysalis leachi* were easily distinguished from all the rest by a peculiar tendency of the filaments to be grouped side by side, by the presence of unusually marked chromophobe areas within the filaments, and by the occurrence of forms indicative of the possibility of multiplication by longitudinal splitting (Figs. 3, 9, and 18).

In *Rhipicephalus appendiculatus* the microorganisms were of particularly small size and were grouped distinctively (Figs. 4, 10, and 14). In *Rhipicephalus evertsi* still another type of grouping was manifest (Figs. 5, 11, and 16), which called to mind certain forms of *Rickettsia lectularia* as described by Hertig and Wolbach[4] and represented in their Figs. 25 and 26, while *Rhipicephalus simus* differed sharply from the two above mentioned species of the same genus by the fact that its contained microorganisms were not grouped in any way other than in diplo formation (Figs. 6, 12, and 17). These microorganisms in *Rhipicephalus simus* more closely resembled those in *Boophilus decoloratus* than those in any of the other five species to which reference has already been made; but they differed from the microorganisms in *Boophilus decoloratus* by their failure to exhibit red- and blue-staining materials and in the absence of branching forms.

On the basis of these observations it would be quite possible to identify unfed larvæ of any of these species by an examination of the contained microorganisms.

In addition, microorganisms having somewhat similar general properties were observed in unfed larvæ of *Argas persicus* and *Ornithodorus megnini*, but differing slightly as between their respective hosts. The failure to detect like microorganisms in alcohol-fixed specimens of *Hyalomma ægyptium*, *Margaropus winthemi*, *Ornithodorus moubata*, and *Rhipicephalus capensis*, as noted in Table I, may have been due to imperfect preservation and consequently cannot be interpreted as conclusive evidence of the absence of microorganisms of this general type in the unfed larvæ of these species.

Evidence of the Hereditary Transmission of the Microorganisms.

That the finding of the microorganisms in the unfed larvæ of eight

[4] Hertig, M., and Wolbach, S. B., *J. Med. Research*, 1924, xliv, 329.

species,[5] in the eggs of ten species,[6] and in smears of the adults of four others,[7] is indicative of hereditary transmission on a fairly large scale is supported by tracing them through the entire life cycle of three species.

Thus, in *Amblyomma hebræum*, *Rhipicephalus evertsi*, and *Rhipicephalus simus* they were observed not only in the Malpighian tubules of unfed larvæ, carefully protected from bacterial contamination, but also in the Malpighian tubules of nymphæ and in the Malpighian tubules and eggs of adult females. The series of preparations of *Amblyomma hebræum* made at close intervals for the study of *Rickettsia ruminantium* was particularly valuable. It was observed that throughout the periods of engorgement and moulting the microorganisms exhibited with constancy the morphological and microchemical properties already alluded to, so that the likelihood of error in their identification was almost negligible.

Occurrence of the Microorganisms.

Although technical difficulties prevented the detection of the microorganisms in all the individual ticks examined, favorable preparations always revealed their presence in 100 per cent of the ticks when prolonged search was made, and it is believed that they were invariably present in the specimens of the following species which were studied: *Amblyomma americana*, *Argas persicus*, *Boophilus decoloratus*, *Dermacentor variabilis*, *Amblyomma hebræum*, *Dermacentor venustus*, *Hæmophysalis leachi*, *Hyalomma ægyptium*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Ornithodorus megnini*, *Ornithodorus turicata*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi*, *Rhipicephalus sanguineus*, and *Rhipicephalus simus*.

The failure to observe them, as indicated by the negative signs (—) in Table I in *Amblyomma maculatum*, *Amblyomma tuberculatum*, *Argas mineatus*, *Dermacentor albipictus*, *Hæmophysalis leporis palustris*, *Margaropus winthemi*, *Ornithodorus*,

[5] *Amblyomma hebræum*, *Argas persicus*, *Boophilus decoloratus*, *Hæmophysalis leachi*, *Ornithodorus megnini*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi*, and *Rhipicephalus simus*.

[6] *Amblyomma americana*, *Amblyomma hebræum*, *Boophilus decoloratus*, *Dermacentor variabilis*, *Dermacentor venustus*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Ornithodorus turicata*, *Rhipicephalus evertsi*, and *Rhipicephalus sanguineus*.

[7] *Argas persicus*, *Rhipicephalus appendiculatus*, *Rhipicephalus pulchellus*, and *Rhipicephalus simus*.

moubata, and *Rhipicephalus capensis* is open to two interpretations, either that they are absent, or that further search in better preparations would bring them to light. In favor of the latter hypothesis may be mentioned the fact that the material available for their study was much less satisfactory than that of the species in which the microorganisms were actually discovered, and, further, the consideration that these ticks all belong to genera other species of which were found to contain the microorganisms.

There is no indication that the season of the year has any influence upon the association of the microoganisms with their arachnid hosts.

TABLE II.

Geographic Distribution of the Microorganisms.

Similar micro-organisms.	In.	Collected at.
Present.	*Amblyomma americana.*	1. Columbia, S. C., by Dr. W. K. Lewis. 2. Washington, N. C., by Dr. Hartwell Robbins. 3. Baton Rouge, La., by Dr. E. Pegram Flower.
"	*Dermacentor variabilis.*	1. Urbana, Ill., by Dr. W. P. Flint. 2. College Park, Md., by Dr. E. N. Cory.
"	*Dermacentor venustus.*	1. Hamilton, Mont., by Dr. R. R. Parker. 2. Wawawai, Wash., by Dr. A. L. Melander.
"	*Margaropus annulatus.*	1. Columbia, S. C., by Dr. W. K. Lewis. 2. Washington, N. C., by Dr. Hartwell Robbins. 3. Baton Rouge, La., by Dr. E. Pegram Flower.
"	*Margaropus annulatus australis.*	1. Jamaica, British West Indies, by Dr. B. E. Washburn. 2. Trinidad, Leeward Islands, by Mr. John L. Rice.
"	*Rhipicephalus sanguineus.*	1. Honolulu, Hawaiian Islands, by Mr. N. H. Cowdry. 2. Onderstepoort, Union of South Africa.

The evidence at hand hardly permits a statement of the geographical distribution of the microorganisms. In other words, it is possible that South African ticks are especially prone to harbor microorganisms of this kind by reason of favorable climatic conditions and other unknown factors and that the same species elsewhere might not possess them. Against this interpretation, however, was the observation of apparently identical microorganisms in individuals of a single

species collected in widely different localities. For convenience, the available information is summarized in Table II.

It will be noted that the majority of the ticks examined came from tropical or temperate regions, but this does not necessarily mean that temperature is in any sense a controlling factor in the distribution of the microorganisms, since they also occur in specimens of *Dermacentor venustus* originating at Hamilton, Montana, where the winter temperature falls far below zero.

From the geographic point of view, Honolulu and Onderstepoort are very widely separated. Not only is their location almost antipodal, but Honolulu is hot and humid and situated at sea-level, while Onderstepoort is at an altitude of about 4,000 feet, somewhat cooler, and very dry in winter, when the specimens were collected. Yet examples of *Rhipicephalus sanguineus* collected at these two places harbor microorganisms which are indistinguishable in their morphology and tinctorial properties, in their location in the tissues, and in the probability of their hereditary transmission.

DISCUSSION.

The exact systematic position of these hereditarily transmitted microorganisms is not easily established, but the possibility of the existence of a relationship to the *Rickettsiæ* and to certain symbionts may be mentioned.

The Gram-negative properties, intracellular habitat, and bacterium-like shape of the microorganisms and their presence in arthropods would perhaps justify their provisional inclusion under the general heading of *Rickettsiæ*.[2] It will be recalled that this characterization of *Rickettsia* was used as a working basis by Hertig and Wolbach[4] in their studies on *Rickettsia*-like microorganisms in insects and that these studies led them to suggest the propriety of still further restricting the term to proved pathogenic microorganisms having the following properties: small size, pleomorphism, slight affinity for aniline dyes, and intracellular habitat. Since the microorganisms under discussion in this paper are of relatively large size, sometimes 0.5μ or more in diameter, and are apparently non-pathogenic, they obviously do not measure up to the requirements of this more exacting definition of *Rickettsia*.

The microorganisms resemble the symbionts of certain blood-feeding flies and lice in size and to some extent in staining reactions but differ from them sharply in so far that they occur in the Malpighian tubules instead of the alimentary tract and by their failure to give rise to the peculiar organ-like structures called mycetomes.

It is difficult to compare them with the symbionts of ticks because the latter have only been very inadequately described in the literature. Thus, Godoy and Pinto[8] in 1922 mentioned the presence of a microorganism in the ovaries of certain species of Brazilian Ixodidæ. They named it "*Ixodisymbionte*" and noted that it was first discovered by R. Koch in *Rhipicephalus*. Unfortunately Godoy and Pinto gave but few criteria for the identification of the microoganisms and their paper was not illustrated. A careful search for an earlier and more complete description by Koch was unsuccessful. The only other reference to symbionts in ticks which I have been able to find was published in the same year by Buchner[9] and consists merely of a few lines in a paper devoted to a general discussion of symbiosis. It is a record of the occurrence of a symbiont within the egg cells and the cells of the Malpighian tubules of an unspecified species of the genus *Ixodes* and is accompanied by two figures.

Although it cannot be said definitely, it does appear probable that the microorganisms described in this paper belong to the same general category as those previously reported by Godoy and Pinto and by Buchner. If the former are correct in their reference to Koch it would seem that this investigator deserves credit not only for his well known discovery of the symbionts of the tsetse flies but also for the first description of symbionts in ticks which we are now able to recognize as very widely distributed in both the Argasidæ and the Ixodidæ.

SUMMARY.

1. Pleomorphic, bacterium-like, Gram-negative, intracellular microorganisms, which stained much less intensely with ordinary dyes than

[8] Godoy, A., and Pinto, C., *Brazil-med.*, 1922, ii, 335; reviewed in *Trop. Dis. Bull.*, 1923, xx, 474.

[9] Buchner, P., *Naturwissenschaften*, 1922, x, 1.

most bacteria were found in sixteen species of ticks comprising examples of the Argasidæ and the Ixodidæ.

2. In six of these species studied intensively slight differences in the microorganisms were detected, sufficient to permit identification of the vectors by microscopic examination of the microorganism alone.

3. No evidence was seen of injury to the tissues of the arachnid hosts of the microorganisms other than that incident to mechanical distention of the cells containing them.

4. The detection of the microorganisms in the eggs of ten species, in the unfed larvæ of eight species, and at nearly related stages throughout the life cycle of three others leads to the conclusion that they are transmitted hereditarily.

EXPLANATION OF PLATES.

PLATE 33.

Photomicrographs of the microorganisms, made with apochromatic objective 3 mm., 1.40 aperture, and compensating ocular 8, giving a magnification of 1,400 diameters, of air-dried smears of unfed larvæ, fixed in absolute alcohol and colored by Giemsa's stain.

FIG. 1. *Amblyomma hebræum.*
FIG. 2. *Boophilus decoloratus.*
FIG. 3. *Hæmophysalis leachi.*
FIG. 4. *Rhipicephalus appendiculatus.*
FIG. 5. *Rhipicephalus evertsi.*
FIG. 6. *Rhipicephalus simus.*

PLATE 34.

Drawings of selected microorganisms from smears of unfed larval ticks to show extent of their pleomorphism. All the drawings were made from air-dried smears, fixed in alcohol and colored by Giemsa's stain with apochromatic objective 1.5 mm., compensating ocular 8, and camera lucida, and have been slightly reduced in reproduction.

FIG. 7. *Amblyomma hebræum.*
FIG. 8. *Boophilus decoloratus.*
FIG. 9. *Hæmophysalis leachi.*
FIG. 10. *Rhipicephalus appendiculatus.*
FIG. 11. *Rhipicephalus evertsi.*
FIG. 12. *Rhipicephalus simus.*

PLATE 35.

All the figures are drawings of portions of the Malpighian tubules of unfed larval ticks made with apochromatic objective 1.5 mm., compensating ocular 8, and camera lucida and have been slightly reduced in reproduction.

FIG. 13. *Boophilus decoloratus.*

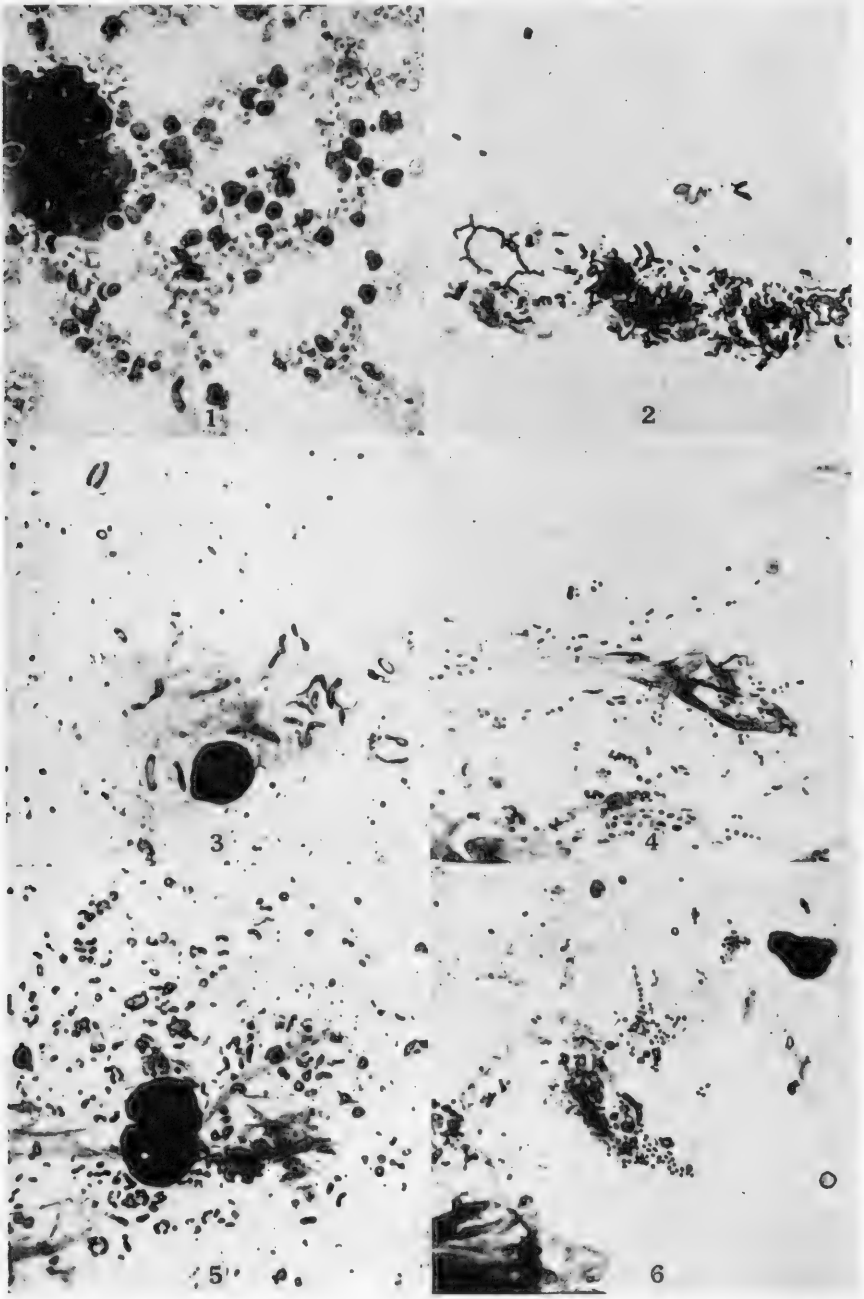
FIG. 14. *Rhipicephalus appendiculatus.*

FIG. 15. *Amblyomma hebræum.*

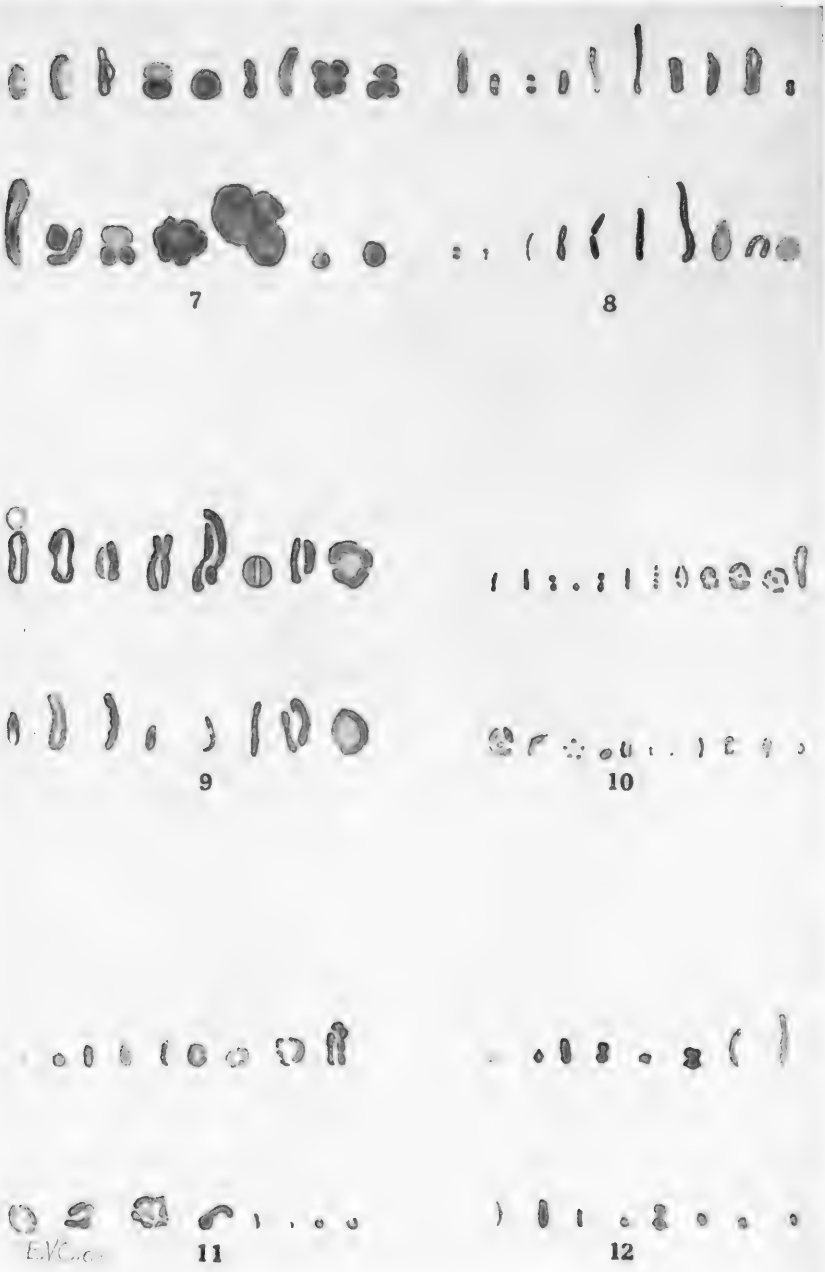
FIG. 16. *Rhipicephalus evertsi.*

FIG. 17. *Rhipicephalus simus.*

FIG. 18. *Hæmophysalis leachi.*



(Cowdry: Microorganisms in ticks.)



(Cowdry: Microorganisms in ticks.)

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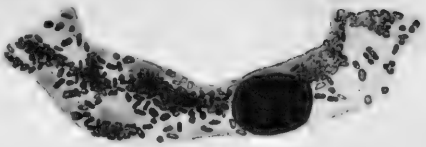

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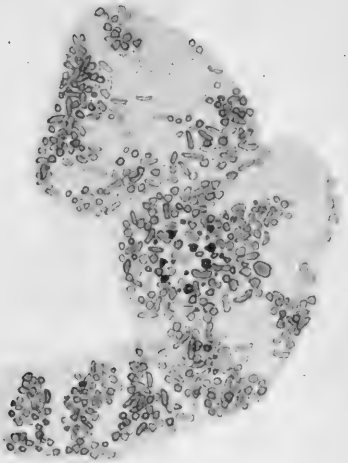

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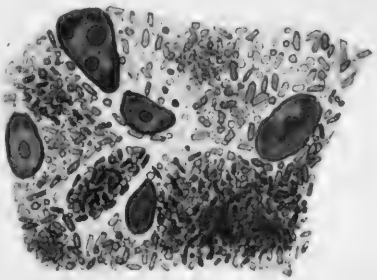

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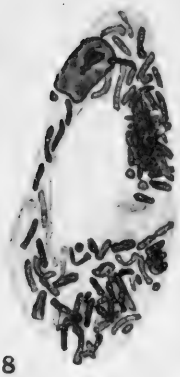

18

(Cowdry: Microorganisms in ticks.)

INDEX TO AUTHORS.

A

Abramson, Harold A. The influence of a low electromotive force on the electrophoresis of lymphocytes of different ages, 445

Amoss, Harold L. The composite nature of a pure culture of a virulent pneumococcus, 649

—. See FLEXNER and AMOSS, 215, 233, 357

Avery, Oswald T. See NEILL and AVERY, 285

B

Binger, Carl A. L. See DAVIES, BROW, and BINGER, 37

Branch, Arnold, and **Stillman, Ernest G.** Pathology of the experimental pneumonias in mice following inhalation of *Streptococcus hæmolyticus*, of Friedländer's bacillus, and of pneumococcus, 631

—. See STILLMAN and BRANCH, 623

Bronfenbrenner, Jacques J., and **Korb, Charles.** Studies on the bacteriophage of d'Hérelle. I. Is the lytic principle volatile? 73

— and **Straub, Elmer L.** The influence of the "diaplyte" antigen of Dreyer on tuberculosis of the guinea pig, 257

Brow, George R. See DAVIES, BROW, and BINGER, 37

Brown, Wade H., and **Pearce, Louise.** The occurrence of stigmata of congenital syphilis in rabbits with especial reference to the factor of inheritance, 795

—. See PEARCE and BROWN, 673

Burke, Victor, and **Skinner, Horace.** Resistance of bacterial spores to the triphenylmethane dyes, 471

C

Chesney, Alan M., and **Kemp, Jarold E.** Studies in experimental syphilis. I. The influence of the size of inoculum on the course of experimental syphilis in the rabbit, 479

— and —. II. The influence of a non-specific inflammatory reaction upon the development of the chancre, 487

Clark, Harry. See HAWKINS and CLARK, 761

Cowdry, E. V. A group of microorganisms transmitted hereditarily in ticks and apparently unassociated with disease, 817

D

Davies, H. Whitridge, Brow, George R., and **Binger, Carl A. L.** The respiratory response to carbon dioxide, 37

Drury, Douglas R., and **Rous, Peyton.** Suppression of bile as a result of impairment of liver function, 611
—. See Rous and Drury, 601

E

Ebeling, Albert H. A pure strain of thyroid cells and its characteristics, 337
Edmunds, Charles W., and **Nelson, Erwin E.** Factors concerned in the polycythemia produced by the subcutaneous injection of epinephrine, 1
Elman, Robert, and **McMaster, Philip D.** Studies on urobilin physiology and pathology. I. The quantitative determination of urobilin, 503
—. See McMaster and Elman, 513, 719

F

Feng, Chih-Tung. See Miles and Feng, 137
Flexner, Simon, and **Amoss, Harold L.** Contributions to the pathology of experimental virus encephalitis. I. An exotic strain of encephalitogenic virus, 215
— and —. II. Herpetic strains of encephalitogenic virus, 233
— and —. III. Varieties and properties of the herpes virus, 357
Florence, Laura. See Smith and Florence, 25
Frühbauer, Emily. See Mackenzie, 53

G

Gorter, E., and **Grendel, F.** On bimolecular layers of lipoids on the chromocytes of the blood, 439
Grendel, F. See Gorter and Grendel, 439

H

Haden, Russell L., and **Orr, Thomas G.** The distribution of chlorides in the blood of the dog after experimental intestinal obstruction, 113
— and —. Experimental high intestinal obstruction in the monkey, 107
— and —. Experimental obstruction of the jejunum. Effect of sodium chloride introduced directly into the lumen of the intestine below the point of obstruction, 707
— and —. The sodium content of the blood of the dog after experimental intestinal obstruction, 119
Hawkins, James A., and **Clark, Harry.** Skin reactions to simultaneous treatments with radiant heat and soft x-rays, 761

Hitchcock, Charles H. Serological relationships among and between the streptococci and pneumococci, 13

J

Jones, F. S. The liver as a source of bacterial agglutinin, 767

K

Kemp, Jarold E. See Chesney and Kemp, 479, 487
Korb, Charles. See Bronfenbrenner and Korb, 73

L

Landsteiner, K., and **van der Scheer, James.** On the antigens of red blood corpuscles. The question of lipoid antigens, 427

Landsteiner, K. See MURPHY and LANDSTEINER, 807

Lewis, Paul A., and **Loomis, Dorothy.** Allergic irritability. II. Anaphylaxis in the guinea pig as affected by the inheritance, 327

Loomis, Dorothy. See LEWIS and LOOMIS, 327

M

Mackenzie, George M. The significance of anaphylaxis in pneumococcus immunity, 53

— and **Woo, Shutai T.** The production and significance of cutaneous allergy to pneumococcus protein, 65

McMaster, Philip D., and **Elman, Robert.** Studies on urobilin physiology and pathology. II. Derivation of urobilin. Relation of the bile to the presence of urobilin in the body, 513

— and —. III. Absorption of pigments of biliary derivation from the intestine, 719

—. See ELMAN and MCMASTER, 503

Miles, Lee Monroe, and **Feng, Chih-Tung.** Calcium and phosphorus metabolism in osteomalacia, 137

Mueller, J. Howard. See ZINSSER and MUELLER, 159

Murphy, James B., and **Landsteiner, K.** Experimental production and transmission of tar sarcomas in chickens, 807

— and **Sturm, Ernest.** A comparison of the effects of x-ray and dry heat on antibody formation, 245

N

Nakahara, Waro. Resistance to spontaneous mouse cancer induced by injections of oleic acid, 347

Neill, James M. Studies on the oxidation-reduction of hemoglobin and methemoglobin. I. The changes induced by pneumococci and by sterile animal tissue, 299

—. II. The oxidation of hemoglobin and reduction of methemoglobin by anaerobic bacilli and by sterile plant tissue, 535

—. III. The formation of methemoglobin during the oxidation of autoxidizable substances, 551

—. IV. The inhibition of "spontaneous" methemoglobin formation, 561

— and **Avery, Oswald T.** Studies on oxidation and reduction by pneumococcus. VIII. Nature of the oxidation-reduction systems in sterile pneumococcus extracts, 285

Nelson, Erwin E. See EDMUNDS and NELSON, 1

du Noüy, P. Lecomte. Surface tension of serum. XII. A technique for the accurate study of the drop in function of the time, 663

—. XIII. Physicochemical studies of immune serum, 779

O

Olitsky, Peter K. Experiments on the cultivation of the active agent of mosaic disease in tobacco and tomato plants, 129

Orcutt, Marion L. See SMITH and ORCUTT, 89

Orr, Thomas G. See HADEN and ORR, 107, 113, 119, 707

P

Pearce, Louise, and **Brown, Wade H.** Distinctive characteristics of infections produced by *Treponema pertenue* in the rabbit, 673

Pearce, Louise. See BROWN and PEARCE, 795

Pritchett, Ida W. Microbic virulence and host susceptibility in paratyphoid-enteritidis infection of white mice. VI. The relative susceptibility of different strains of mice to *per os* infection with the Type II bacillus of mouse typhoid (*Bacillus pestis caviæ*), 195

—. VII. Seasonal variation in the susceptibility of different strains of mice to *per os* infection with the Type II bacillus of mouse typhoid (*Bacillus pestis caviæ*), 209

R

Reimann, Hobart A. Variations in specificity and virulence of pneumococci during growth *in vitro*, 587

Rivers, Thomas M. Skin infection of rabbits with hemolytic streptococci isolated from a patient with erysipelas. I. Method of demonstrating protective action of immune sera, 179

— and **Tillett, William S.** Local passive immunity in the skin of rabbits to infection with (1) a filterable virus, and (2) hemolytic streptococci, 185

Rous, Peyton. The relative reaction within living mammalian tissues. I. General features of vital staining with litmus, 379

—. II. On the mobilization of acid material within cells, and the reaction as influenced by the cell state, 399

—. III. Indicated differences in the reaction of the blood and tissues on vital staining with phthaleins, 451

Rous, Peyton. IV. Indicated differences in the reaction of the organs on vital staining with phthaleins, 739

— and **Drury, Douglas R.** Jaundice as an expression of the physiological wastage of corpuscles, 601

—. See DRURY and ROUS, 611

S

Skinner, Horace. See BURKE and SKINNER, 471

Smith, David T., and **Webster, Leslie T.** Epidemiological studies on respiratory infections of the rabbit. VI. Etiology of otitis media, 275

Smith, Theobald. Focal interstitial nephritis in the calf following interference with the normal intake of colostrum, 413

—. Hydropic stages in the intestinal epithelium of new-born calves, 81

—. Pneumonia associated with *Bacillus abortus* (Bang) in fetuses and new-born calves, 639

— and **Florence, Laura.** *Encephalitozoon cuniculi* as a kidney parasite in the rabbit, 25

— and **Orcutt, Marion L.** The bacteriology of the intestinal tract of young calves with special reference to the early diarrhea ("scours"), 89

Stillman, Ernest G., and **Branch, Arnold.** Experimental pneumonia in mice following the inhalation of *Streptococcus hæmolyticus* and of Friedländer's bacillus, 623

—. See BRANCH and STILLMAN, 631

Straub, Elmer L. See BRONFENBRENNER and STRAUB, 257

Sturm, Ernest. See MURPHY and STURM, 245

T

Tillett, William S. See RIVERS and TILLETT, 185

V

Van Allen, Chester M. The relationship between the growth capacity of primary tumors and the distribution of metastases of a transplanted tumor of the rabbit, 691

van der Scheer, James. See LANDSTEINER and VAN DER SCHEER, 427

W

Webster, Leslie T. Biology of *Bacterium lepisepticum.* I. Effects of oxygen tension and the presence of rabbit blood on growth, dissociation, and virulence, 571

—. See SMITH and WEBSTER, 275

White, A. C. The bicarbonate reserve and the dissociation curve of oxyhemoglobin in febrile conditions, 315

Woo, Shutai T. See MACKENZIE and Woo, 65

Z

Zinsser, Hans, and **Mueller, J. Howard.** On the nature of bacterial allergies, 159

INDEX TO SUBJECTS.

A

Abortus:
Bacillus, pneumonia in fetus (SMITH) 639
—, — — new-born calf (SMITH) 639
Absorption:
Bile pigment, from intestine (MCMASTER and ELMAN) 719
Acid:
Cell, acid material (ROUS) 399
Agglutinin:
Bacterial, liver as source (JONES) 767
Allergy:
Bacterial (ZINSSER and MUELLER) 159
Irritability. II (LEWIS and LOOMIS) 327
Skin, to pneumococcus protein (MACKENZIE and WOO) 65
Anaerobe:
Bacilli, oxidation of hemoglobin (NEILL) 535
—, reduction of methemoglobin (NEILL) 535
Anaphylaxis:
Heredity, influence of, on (LEWIS and LOOMIS) 327
Pneumococcus immunity (MACKENZIE) 53
Antibody:
Formation, x-rays and dry heat, comparison of effects (MURPHY and STURM) 245
Antigen:
Diaplyte, of Dreyer, influence on tuberculosis (BRONFENBRENNER and STRAUB) 257
Erythrocyte (LANDSTEINER and VAN DER SCHEER) 427
Lipoid (LANDSTEINER and VAN DER SCHEER) 427
Autoxidation:
Methemoglobin formation during (NEILL) 551

B

Bacillus:
abortus pneumonia in fetus (SMITH) 639
— — — new-born calf (SMITH) 639
Anaerobic, oxidation of hemoglobin (NEILL) 535
—, reduction of methemoglobin (NEILL) 535
Friedländer's, pneumonia after inhalation (STILLMAN and BRANCH) 623
—, — — —, pathology (BRANCH and STILLMAN) 631
pestis caviæ, *per os* infection, seasonal variation in susceptibility (PRITCHETT) 209
— —, — — infection, susceptibility (PRITCHETT) 195
Typhoid, mouse, *per os* infection, seasonal variation in susceptibility (PRITCHETT) 209

Bacillus—*continued:*
Typhoid, mouse, *per os* infection, susceptibility (PRITCHETT) 195
Bacteriology:
Intestinal tract, early diarrhea in calf (SMITH and ORCUTT) 89
— —, scours in calf (SMITH and ORCUTT) 89
Bacteriophage:
Twort-d'Hérelle phenomenon. I (BRONFENBRENNER and KORB) 73
Bacterium:
Agglutinin, liver as source (JONES) 767
Allergy (ZINSSER and MUELLER) 159
lepisepticum, biology. I (WEBSTER) 571
Spore, resistance to triphenylmethane dyes (BURKE and SKINNER) 471
Bicarbonate:
Reserve during pyrexia (WHITE) 315
Bile:
Pigment, absorption from intestine (MCMASTER and ELMAN) 719
Suppression as result of impairment of liver function (DRURY and ROUS) 611
Urobilin in body, relation (MCMASTER and ELMAN) 513
Biology:
Bacterium lepisepticum. I (WEBSTER) 571
Blood:
Bacterium lepisepticum dissociation, effect on, of (WEBSTER) 571
— — growth, effect on, of (WEBSTER) 571
— — virulence, effect on, of (WEBSTER) 571
Blood—*continued:*
Cell, red. *See* Erythrocyte.
Chlorides, after intestinal obstruction (HADEN and ORR) 113
Chromocyte, bimolecular layers of lipoids (GORTER and GRENDEL) 439
Corpuscle, red. *See* Erythrocyte.
Reaction indicated by vital staining with phthaleins (ROUS) 451
Sodium content after intestinal obstruction (HADEN and ORR) 119

C

Calcium:
Metabolism in osteomalacia (MILES and FENG) 137
Carbon dioxide:
Respiratory response (DAVIES, BROW, and BINGER) 37
Carcinoma:
Spontaneous, resistance induced by injections of oleic acid (NAKAHARA) 347
Cell:
Acid material (ROUS) 399
Blood, red. *See* Erythrocyte.
Reaction (ROUS) 399
Thyroid, pure strain (EBELING) 337
Chancre:
Inflammation, non-specific, influence on development of (CHESNEY and KEMP) 487
Chloride(s):
Blood, after intestinal obstruction (HADEN and ORR) 113
Chromocyte:
Blood, bimolecular layers of lipoids (GORTER and GRENDEL) 439

Colostrum:
Nephritis, interstitial, in calf after interference with normal intake (SMITH) 413

Corpuscle:
Blood, red. *See* Erythrocyte.
Physiological wastage expressed as jaundice (ROUS and DRURY) 601

Cultivation:
Mosaic disease in tobacco plant (OLITSKY) 129
— — — tomato plant (OLITSKY) 129
Pneumococcus, virulent (AMOSS) 649

Cuniculi:
Encephalitozoon, in kidney of rabbit (SMITH and FLORENCE) 25

Cutaneous:
See Skin.

D

Diarrhea:
Calf, young, bacteriology of intestinal tract (SMITH and ORCUTT) 89

Dissociation:
Bacterium lepisepticum, effect of blood (WEBSTER) 571
— —, — — oxygen tension (WEBSTER) 571
Oxyhemoglobin during pyrexia (WHITE) 315

Dyes:
Triphenylmethane, resistance of bacterial spores (BURKE and SKINNER) 471

E

Electromotive force:
Electrophoresis of lymphocytes, influence on, of (ABRAMSON) 445

Electrophoresis:
Lymphocyte, influence of electromotive force (ABRAMSON) 445

Encephalitis:
Virus, exotic strain (FLEXNER and AMOSS) 215
—, herpetic strains (FLEXNER and AMOSS) 233
—, pathology. I (FLEXNER and AMOSS) 215
—, —. II (FLEXNER and AMOSS) 233
—, —. III (FLEXNER and AMOSS) 357

Encephalitozoon:
cuniculi in kidney of rabbit (SMITH and FLORENCE) 25

Enteritidis:
Paratyphoid-, infection, microbic virulence and host susceptibility. VI (PRITCHETT) 195
—, —, — — and host susceptibility. VII (PRITCHETT) 209

Epidemiology:
Respiratory infection of rabbit. VI (SMITH and WEBSTER) 275

Epinephrine:
Polycythemia produced by subcutaneous injection (EDMUNDS and NELSON) 1

Epithelium: (*See also* Skin.)
Intestinal, hydropic, of newborn calf (SMITH) 81

Erysipelas:
Skin infection with hemolytic streptococcus from. I (RIVERS) 179

Erythrocyte:
Antigen (LANDSTEINER and VAN DER SCHEER) 427

Etiology:
Otitis media (SMITH and WEBSTER) 275
Extract:
Pneumococcus, sterile, oxidation-reduction systems (NEILL and AVERY) 285

F

Fetus:
Bacillus abortus pneumonia (SMITH) 639
Fever:
Bicarbonate reserve (WHITE) 315
Oxyhemoglobin dissociation (WHITE) 315
Filtration:
Virus, filterable, local passive immunity in infected skin (RIVERS and TILLETT) 185
Friedländer:
Bacillus. *See* Bacillus.

G

Gland:
Thyroid. *See* Thyroid.
Growth:
Bacterium lepisepticum, effect of blood (WEBSTER) 571
— —, — — oxygen tension (WEBSTER) 571
Pneumococcus *in vitro*, specificity and virulence (REIMANN) 587
Tumor, primary, growth capacity, relation to distribution of metastases (VAN ALLEN) 691

H

Hæmolyticus:
Streptococcus, pneumonia after inhalation (STILLMAN and BRANCH) 623
—, — — —, pathology (BRANCH and STILLMAN) 631
Heat:
Dry, antibody formation, comparison with effect of x-rays (MURPHY and STURM) 245
Radiant, skin reaction to treatment with soft x-rays and (HAWKINS and CLARK) 761
Hemoglobin:
Oxidation by anaerobic bacilli (NEILL) 535
— — sterile plant tissue (NEILL) 535
Oxidation-reduction of hemoglobin and methemoglobin. I (NEILL) 299
— — — and methemoglobin. II (NEILL) 535
— — — and methemoglobin. III (NEILL) 551
— — — and methemoglobin. IV (NEILL) 561
Hemolysis:
Streptococcus, hemolytic, from erysipelas, skin infection. I (RIVERS) 179
—, —, local passive immunity in infected skin (RIVERS and TILLETT) 185
Heredity:
Anaphylaxis, influence on, of (LEWIS and LOOMIS) 327
Microorganism hereditarily

Heredity—*continued:*
transmitted in ticks and unassociated with disease (COWDRY) 817
Syphilis, congenital, stigmata with reference to (BROWN and PEARCE) 795
d'Hérelle phenomenon:
See Twort-d'Hérelle phenomenon.
Herpes:
Virus (FLEXNER and AMOSS) 357
— inducing encephalitis (FLEXNER and AMOSS) 233
Host:
Susceptibility and microbic virulence in paratyphoid-enteritidis infection. VI (PRITCHETT) 195
— — — — in paratyphoid-enteritidis infection. VII (PRITCHETT) 209
Hydropic:
Intestinal epithelium of newborn calf (SMITH) 81

I

Immunity:
Pneumococcus, anaphylaxis (MACKENZIE) 53
Serum, immune, physicochemistry (DU NOÜY) 779
Streptococcus, hemolytic, from erysipelas, protective action of immune serum against skin infection (RIVERS) 179
—, —, local passive immunity in infected skin (RIVERS and TILLETT) 185
Virus, filterable, local passive immunity in infected skin (RIVERS and TILLETT) 185
Inflammation:
Non-specific, influence on development of chancre (CHESNEY and KEMP) 487
Interstitial:
Nephritis, in calf after interference with normal intake of colostrum (SMITH) 413
Intestine:
Bile pigment absorption (McMASTER and ELMAN) 719
Diarrhea, early, bacteriology of intestinal tract of calf (SMITH and ORCUTT) 89
Epithelium, hydropic, of newborn calf (SMITH) 81
Obstruction (HADEN and ORR) 107
—, blood chlorides (HADEN and ORR) 113
— of jejunum, effect of sodium chloride introduced into lumen below obstruction (HADEN and ORR) 707
—, sodium content of blood (HADEN and ORR) 119
Scours, bacteriology of intestinal tract of calf (SMITH and ORCUTT) 89
In vitro:
Pneumococcus, specificity and virulence during growth (REIMANN) 587
Irritability:
Allergic. II (LEWIS and LOOMIS) 327

J

Jaundice:
Physiological wastage of corpuscles expressed as (ROUS and DRURY) 601

Jejunum:
Obstruction (HADEN and ORR) 707

K

Kidney:
Encephalitozoon cuniculi in kidney of rabbit (SMITH and FLORENCE) 25

L

Lepisepticum:
Bacterium, biology. I (WEBSTER) 571
Lipoid:
Antigen (LANDSTEINER and VAN DER SCHEER) 427
Bimolecular layers on chromocytes of blood (GORTER and GRENDEL) 439
Litmus:
Vital staining (ROUS) 379
Liver:
Agglutinin, bacterial, liver as source (JONES) 767
Function, impaired, resulting in suppression of bile (DRURY and ROUS) 611
Lymphocyte:
Electrophoresis, influence of electromotive force (ABRAMSON) 445
Lysis:
Transmissible, volatility of principle (BRONFENBRENNER and KORB) 73

M

Mammal:
Tissue, living, relative reaction. I (ROUS) 379
—, —, — —. II (ROUS) 399
—, —, — —. III (ROUS) 451
Mammal—*continued:*
Tissue, living, relative reaction. IV (ROUS) 739
Metabolism:
Calcium, in osteomalacia (MILES and FENG) 137
Phosphorus, in osteomalacia (MILES and FENG) 137
Metastasis:
Tumor, distribution, relation to growth capacity of primary tumor (VAN ALLEN) 691
Methemoglobin:
Formation during oxidation of autoxidizable substances (NEILL) 551
—, spontaneous, inhibited (NEILL) 561
Oxidation-reduction of hemoglobin and. I (NEILL) 299
— — — —. II (NEILL) 535
— — — —. III (NEILL) 551
— — — —. IV (NEILL) 561
Reduction by anaerobic bacilli (NEILL) 535
— — sterile plant tissue (NEILL) 535
Microbe:
Virulence and host susceptibility in paratyphoid-enteritidis infection. VI (PRITCHETT) 195
— — — — in paratyphoid-enteritidis infection. VII (PRITCHETT) 209
Microorganism:
Tick, transmitted hereditarily and unassociated with disease (COWDRY) 817
Mosaic disease:
Cultivation in tobacco plant (OLITSKY) 129

Mosaic disease—*continued:*
Cultivation in tomato plant (OLITSKY) 129
Mouth:
Bacillus pestis caviæ, per os infection, seasonal variation in susceptibility (PRITCHETT) 209
— — —, *per os* infection, susceptibility (PRITCHETT) 195
Typhoid, mouse, *per os* infection with bacillus, seasonal variation in susceptibility (PRITCHETT) 209
—, —, — — infection with bacillus, susceptibility (PRITCHETT) 195

N

Nephritis:
Interstitial, in calf after interference with normal intake of colostrum (SMITH) 413

O

Obstruction:
Intestine (HADEN and ORR) 107
—, blood chlorides (HADEN and ORR) 113
—, sodium content of blood (HADEN and ORR) 119
Jejunum (HADEN and ORR) 707
Sodium chloride introduced into lumen of intestine below (HADEN and ORR) 707
Oleic acid:
Carcinoma, spontaneous, resistance induced by (NAKAHARA) 347
Organ:
Reaction indicated by vital staining with phthaleins (ROUS) 739
Osteomalacia:
Metabolism, calcium (MILES and FENG) 137
—, phosphorus (MILES and FENG) 137
Otitis:
Media, etiology (SMITH and WEBSTER) 275
Oxidation:
Hemoglobin by anaerobic bacilli (NEILL) 535
— — sterile plant tissue (NEILL) 535
Methemoglobin formation during oxidation of autoxidizable substances (NEILL) 551
Reduction and, by pneumococcus. VIII (NEILL and AVERY) 285
-reduction of hemoglobin and methemoglobin. I (NEILL) 299
— — — and methemoglobin. II (NEILL) 535
— — — and methemoglobin. III (NEILL) 551
— — — and methemoglobin. IV (NEILL) 561
— systems in sterile pneumococcus extracts (NEILL and AVERY) 285
Oxygen:
Tension, effect on dissociation of *Bacterium lepisepticum* (WEBSTER) 571
—, — — growth of *Bacterium lepisepticum* (WEBSTER) 571
—, — — virulence of *Bacterium lepisepticum* (WEBSTER) 571
Oxyhemoglobin:
Dissociation during pyrexia (WHITE) 315

P

Parasite:
Encephalitozoon cuniculi in kidney of rabbit (SMITH and FLORENCE) 25
Paratyphoid:
-enteritidis infection, microbic virulence and host susceptibility. VI (PRITCHETT) 195
— —, — — and host susceptibility. VII (PRITCHETT) 209
Pathology:
Pneumonia after inhalation of Friedländer's bacillus (BRANCH and STILLMAN) 631
— — — — pneumococcus (BRANCH and STILLMAN) 631
— — — — *Streptococcus hæmolyticus* (BRANCH and STILLMAN) 631
Urobilin. I (ELMAN and McMASTER) 503
—. II (McMASTER and ELMAN) 513
—. III (McMASTER and ELMAN) 719
Virus encephalitis. I (FLEXNER and AMOSS) 215
— —. II (FLEXNER and AMOSS) 233
— —. III (FLEXNER and AMOSS) 357
Pertenue:
Treponema, infection, distinctive characteristics (PEARCE and BROWN) 673
Pestis caviæ:
Bacillus, per os infection, seasonal variation in susceptibility (PRITCHETT) 209
—, — — —, susceptibility (PRITCHETT) 195
Phosphorus:
Metabolism in osteomalacia (MILES and FENG) 137
Phthalein(s):
Vital staining to indicate reaction of blood (ROUS) 451
— — — — reaction of organs (ROUS) 739
— — — — reaction of tissues (ROUS) 451
Physiology:
Corpuscle wastage expressed as jaundice (ROUS and DRURY) 601
Urobilin. I (ELMAN and McMASTER) 503
—. II (McMASTER and ELMAN) 513
—. III (McMASTER and ELMAN) 719
Pigment:
Bile, absorption from intestine (McMASTER and ELMAN) 719
Plant:
Mosaic disease cultivated in tobacco (OLITSKY) 129
— — — — tomato (OLITSKY) 129
Tissue, sterile, oxidation of hemoglobin (NEILL) 535
—, —, reduction of methemoglobin (NEILL) 535
Pneumococcus:
Immunity, anaphylaxis (MACKENZIE) 53
Oxidation and reduction by. VIII (NEILL and AVERY) 285
Oxidation-reduction of hemoglobin and methemoglobin (NEILL) 299
— systems in sterile extracts (NEILL and AVERY) 285

Pneumococcus—*continued:*
Pneumonia after inhalation, pathology (BRANCH and STILLMAN) 631
Protein, cutaneous allergy (MACKENZIE and WOO) 65
Serological relations between streptococcus and (HITCHCOCK) 13
Specificity and virulence during growth *in vitro* (REIMANN) 587
Virulent, pure culture (AMOSS) 649

Pneumonia:
Bacillus abortus, in fetus (SMITH) 639
— —, — new-born calf (SMITH) 639
—, Friedländer's, after inhalation (STILLMAN and BRANCH) 623
—, —, — —, pathology (BRANCH and STILLMAN) 631
Pneumococcus, after inhalation, pathology (BRANCH and STILLMAN) 631
Streptococcus hæmolyticus, after inhalation (STILLMAN and BRANCH) 623
— —, — —, pathology (BRANCH and STILLMAN) 631

Polycythemia:
Production by subcutaneous injection of epinephrine (EDMUNDS and NELSON) 1

Protection:
Hemolytic streptococcus from erysipelas, skin infection, protective action of immune serum (RIVERS) 179

Protein:
Pneumococcus, cutaneous allergy (MACKENZIE and WOO) 65

R

Reduction:
Methemoglobin by anaerobic bacilli (NEILL) 535
— — sterile plant tissue (NEILL) 535
Oxidation and, by pneumococcus. VIII (NEILL and AVERY) 285
Oxidation-, of hemoglobin and methemoglobin. I (NEILL) 299
—, — — and methemoglobin. II (NEILL) 535
—, — — and methemoglobin. III (NEILL) 551
—, — — and methemoglobin. IV (NEILL) 561
—, systems in sterile pneumococcus extracts (NEILL and AVERY) 285

Renal:
See Kidney.

Resistance:
Carcinoma, spontaneous, resistance induced by injections of oleic acid (NAKAHARA) 347

Respiration:
Carbon dioxide, respiratory response (DAVIES, BROW, and BINGER) 37
Infection of rabbit, epidemiology. VI (SMITH and WEBSTER) 275

Roentgen rays:
Antibody formation, comparison with effect of dry heat (MURPHY and STURM) 245

Roentgen rays—*continued:*
Skin reaction to treatment with radiant heat and (HAWKINS and CLARK) 761

S

Sarcoma:
Tar, production in chicken (MURPHY and LANDSTEINER) 807
—, transmission in chicken (MURPHY and LANDSTEINER) 807

Scours:
Bacteriology of intestinal tract of calf (SMITH and ORCUTT) 89

Serology:
Streptococcus and pneumococcus (HITCHCOCK) 13

Serum:
Immune, physicochemistry (DU NOÜY) 779
—, protective action against skin infection with hemolytic streptococcus from erysipelas (RIVERS) 179
Surface tension. XII (DU NOÜY) 663
— —. XIII (DU NOÜY) 779

Skin: (*See also* Epithelium.)
Allergy to pneumococcus protein (MACKENZIE and WOO) 65
Infection with hemolytic streptococcus from erysipelas. I (RIVERS) 179
Reaction to treatment with radiant heat and soft x-rays (HAWKINS and CLARK) 761
Streptococcus, hemolytic, infection, local passive immunity (RIVERS and TILLETT) 185
Virus, filterable, infection, local passive immunity (RIVERS and TILLETT) 185

Sodium:
Blood, after intestinal obstruction (HADEN and ORR) 119

Sodium chloride:
Obstruction of jejunum, effect of sodium chloride introduced into lumen of intestine below obstruction (HADEN and ORR) 707

Specificity:
Inflammation, non-specific, influence on development of chancre (CHESNEY and KEMP) 487
Pneumococcus, virulence and, during growth *in vitro* (REIMANN) 587

Spore:
Bacterial, resistance to triphenylmethane dyes (BURKE and SKINNER) 471

Staining:
Vital, with litmus (ROUS) 379
—, — phthaleins, to indicate reaction of blood (ROUS) 451
—, — —, to indicate reaction of organs (ROUS) 739
—, — —, to indicate reaction of tissues (ROUS) 451

Sterilization:
Hemoglobin and methemoglobin, oxidation-reduction by sterile animal tissue (NEILL) 299
— oxidized by sterile plant tissue (NEILL) 535
Methemoglobin reduced by sterile plant tissue (NEILL) 535
Pneumococcus extracts, sterile, oxidation-reduction systems (NEILL and AVERY) 285

Stigma:
Syphilis, congenital, with refer-

Stigma—*continued:*
ence to inheritance (BROWN and PEARCE 795
Streptococcus:
hæmolyticus pneumonia after inhalation (STILLMAN and BRANCH) 623
— — — —, pathology (BRANCH and STILLMAN) 631
Hemolytic, from erysipelas, skin infection. I (RIVERS) 179
—, local passive immunity in infected skin (RIVERS and TILLETT) 185
Serological relationships between pneumococcus and (HITCHCOCK) 13
Susceptibility:
Bacillus pestis caviæ, per os infection (PRITCHETT) 195
— — —, *per os* infection, seasonal variation (PRITCHETT) 209
Host, and microbic virulence in paratyphoid-enteritidis infection. VI (PRITCHETT) 195
—, — — — in paratyphoid-enteritidis infection. VII (PRITCHETT) 209
Typhoid, mouse, *per os* infection with bacillus (PRITCHETT) 195
—, —, — — infection with bacillus, seasonal variation (PRITCHETT) 209
Syphilis:
I (CHESNEY and KEMP) 479
II (CHESNEY and KEMP) 487
Syphilis—*continued:*
Congenital, stigmata with reference to inheritance (BROWN and PEARCE) 795
Inoculum, influence of size on (CHESNEY and KEMP) 479

T

Tension:
Oxygen, effect on dissociation of *Bacterium lepisepticum* (WEBSTER) 571
—, — — growth of *Bacterium lepisepticum* (WEBSTER) 571
—, — — virulence of *Bacterium lepisepticum* (WEBSTER) 571
Surface, of serum. XII (DU NOÜY) 663
—, — —. XIII (DU NOÜY) 799
Thyroid:
Cells, pure strain (EBELING) 337
Tick:
Microorganism transmitted hereditarily and unassociated with disease (COWDRY) 817
Tissue:
Animal, sterile, oxidation-reduction of hemoglobin and methemoglobin (NEILL) 299
Mammalian, living, relative reaction. I (ROUS) 379
—, —, — —. II (ROUS) 399
—, —, — —. III (ROUS) 451
—, —, — —. IV (ROUS) 739

Tissue—*continued:*
Plant, sterile, oxidation of hemoglobin (NEILL) 535
—, —, reduction of methemoglobin (NEILL) 535
Reaction indicated by vital staining with phthaleins (ROUS) 451
Tobacco:
Mosaic disease, cultivation (OLITSKY) 129
Tomato:
Mosaic disease, cultivation (OLITSKY) 129
Transmission:
Microorganisms, hereditary, in ticks, unassociated with disease (COWDRY) 817
Transplantation:
Tumor, relation of growth capacity of primary tumor to distribution of metastases (VAN ALLEN) 691
Treponema:
pertenue infection, distinctive characteristics (PEARCE and BROWN) 673
Triphenylmethane dyes:
Bacterial spores, resistance to (BURKE and SKINNER) 471
Tuberculosis:
Antigen, diaplyte, of Dreyer, influence of, on (BRONFENBRENNER and STRAUB) 257
Tumor:
Primary, growth capacity, relation to distribution of metastases (VAN ALLEN) 691
Transplanted, relation of growth capacity of primary tumor to distribution of metastases (VAN ALLEN) 691
Twort-d'Hérelle phenomenon:
Bacteriophage. I (BRONFENBRENNER and KORB) 73
Typhoid:
Mouse, bacillus, *per os* infection, seasonal variation in susceptibility (PRITCHETT) 209
—, —, — — infection, susceptibility (PRITCHETT) 195

U

Urobilin:
Bile, relation to presence in body of (MCMASTER and ELMAN) 513
Derivation (MCMASTER and ELMAN) 513
Pathology. I (ELMAN and MCMASTER) 503
—. II (MCMASTER and ELMAN) 513
—. III (MCMASTER and ELMAN) 719
Physiology. I (ELMAN and MCMASTER) 503
—. II (MCMASTER and ELMAN) 513
—. III (MCMASTER and ELMAN) 719
Quantitative determination (ELMAN and MCMASTER) 503

V

Virulence:
Bacterium lepisepticum, effect of blood (WEBSTER) 571

Virulence—*continued:*
Bacterium lepisepticum, effect of oxygen tension (WEBSTER) 571
Microbic, and host susceptibility in paratyphoid-enteritidis infection. VI (PRITCHETT) 195
—, — — — in paratyphoid-enteritidis infection. VII (PRITCHETT) 209
Pneumococcus, specificity and, during growth *in vitro* (REIMANN) 587
—, virulent, pure culture (AMOSS) 649
Virus:
Encephalitis, exotic strain (FLEXNER and AMOSS) 215
—, herpetic strains (FLEXNER and AMOSS) 233
—, pathology. I (FLEXNER and AMOSS) 215
Virus—*continued:*
Encephalitis, pathology. II (FLEXNER and AMOSS) 233
—, —. III (FLEXNER and AMOSS) 357
Filterable, local passive immunity in infected skin (RIVERS and TILLETT) 185
Herpes (FLEXNER and AMOSS) 357
Vital staining:
Litmus (ROUS) 379
Phthalein, to indicate reaction of blood (ROUS) 451
—, — — reaction of organs (ROUS) 739
—, — — reaction of tissues (ROUS) 451

X

X-rays:
See Roentgen rays.

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CONTENTS

VAN ALLEN, CHESTER M. The relationship between the growth capacity of primary tumors and the distribution of metastases of a transplanted tumor of the rabbit..... 691

HADEN, RUSSELL L., and ORR, THOMAS G. Experimental obstruction of the jejunum. Effect of sodium chloride introduced directly into the lumen of the intestine below the point of obstruction..... 707

MCMASTER, PHILIP D., and ELMAN, ROBERT. Studies on urobilin physiology and pathology. III. Absorption of pigments of biliary derivation from the intestine..... 719

ROUS, PEYTON. The relative reaction within living mammalian tissues. IV. Indicated differences in the reaction of the organs on vital staining with phthaleins..... 739

HAWKINS, JAMES A., and CLARK, HARRY. Skin reactions to simultaneous treatments with radiant heat and soft x-rays..... 761

JONES, F. S. The liver as a source of bacterial agglutinin..... 767

DU NOÜY, P. LECOMTE. Surface tension of serum. XIII. On certain physico-chemical changes in serum as a result of immunization. Plates 25 and 26. 779

BROWN, WADE H., and PEARCE, LOUISE. The occurrence of stigmata of congenital syphilis in rabbits with especial reference to the factor of inheritance. Plates 27 to 29..... 795

MURPHY, JAMES B., and LANDSTEINER, K. Experimental production and transmission of tar sarcomas in chickens. Plates 30 to 32..... 807

COWDRY, E. V. A group of microorganisms transmitted hereditarily in ticks and apparently unassociated with disease. Plates 33 to 35..... 817

INDEX TO VOLUME XLI..... 831

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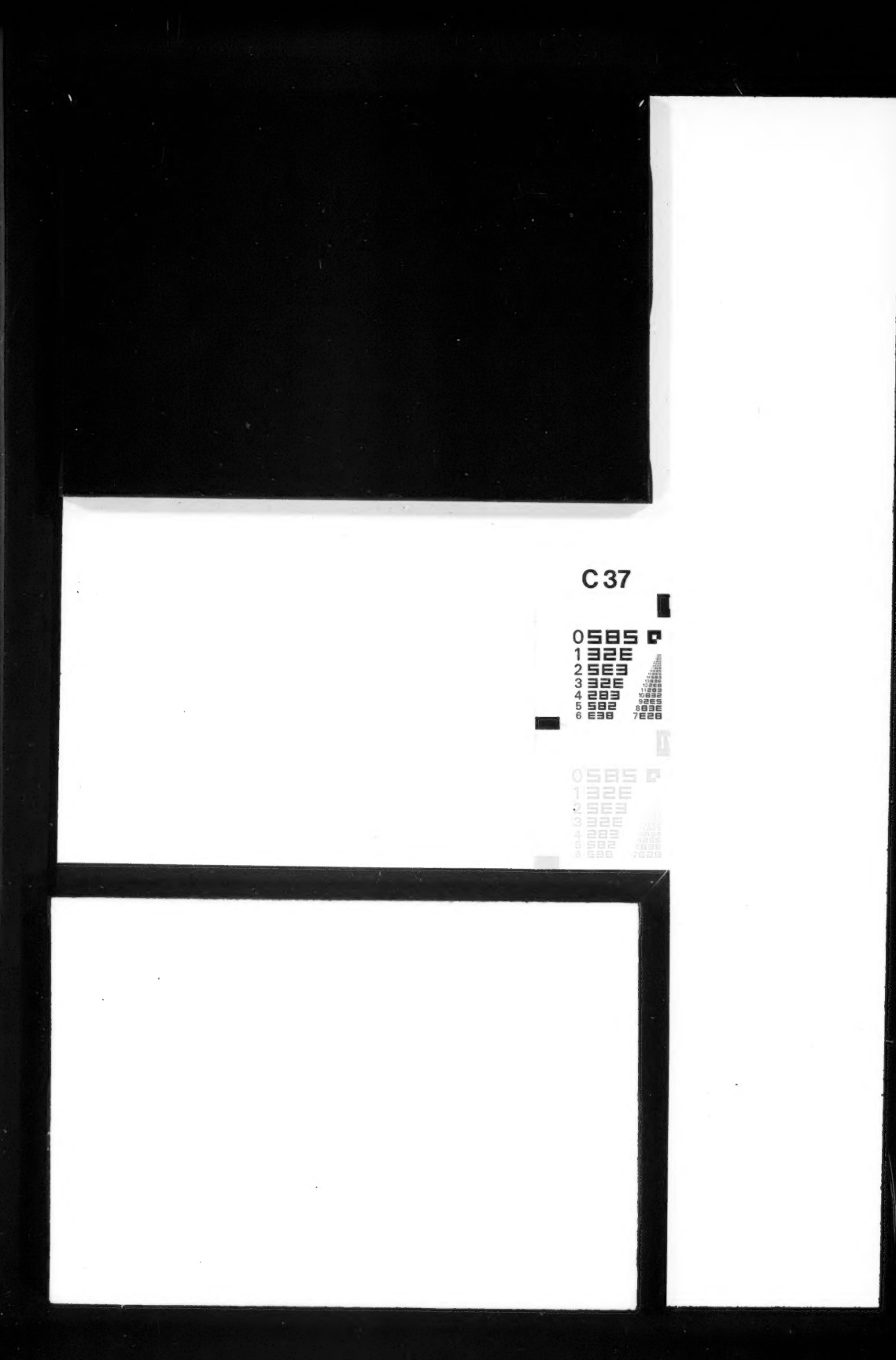
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